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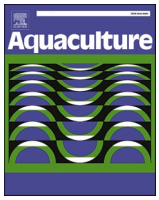

Physiological and transcriptomic analyses of the spotted sea bass (*Lateolabrax maculatus*) in response to low-temperature stress

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ABSTRACT

Spotted sea bass (*Lateolabrax maculatus*) is an economically significant aquaculture species in China. However, its sensitivity to low-temperature stress restricts farming expansion, reduces production efficiency during winter, and increases aquaculture risks. Low-temperature stress poses a significant challenge to aquatic organisms, particularly in aquaculture systems. In this study, we investigated the physiological and molecular responses of spotted sea bass under cold stress conditions. Liver plays a central role in metabolism and immune regulation, maintain energy balance and homeostasis under cold exposure. Serum biochemical analysis revealed significant alterations in key measures related to liver function, metabolism processes, hormone regulation and oxidative stress. These alterations indicated physiological disturbance in spotted sea bass under low-temperature stress. Transcriptome analysis of liver identified differentially expressed genes (DEGs) enriched in pathways such as p53 signaling, ferroptosis, and immune regulation, showing their roles in cold stress responses. Low-temperature stress may result in cell cycle arrest, apoptosis, and iron-dependent oxidative damage, contributing to tissue injury due to the upregulation of p53 and ferroptosis-related genes. At the same time, immune-related pathways, including the RIG-I-like receptor signaling pathway, were suppressed, indicating that low-temperature stress even impaired immune defense mechanisms. These results elucidate the physiological and molecular responses to low-temperature stress in spotted sea bass, particularly involving oxidative stress, endocrine changes, and gene expression dynamics. Our findings offer possible strategies for enhancing cold tolerance and immune defense in aquaculture species, which can reduce farming risks in cold environments.

1. Introduction

Water temperature, a key environmental factor, influences fish in aquaculture systems, particularly in regions where seasonal temperature variations are significant(Watson et al., 2018). As ectothermic organisms, fish are highly susceptible to changes in ambient temperature, and exposure to temperatures under their thermal tolerance range could disrupt wide ranges of physiological disturbances, leading to impaired growth, weakened immune responses, metabolic dysfunction, reduced swimming ability, and, in extreme cases, mortality(Avunje et al., 2013; Islam et al., 2020; Ndong et al., 2007; Szekeres et al., 2014). Extreme cold events in winter were more frequently in recent years. A large range of fish species were susceptible to extreme cold weather caused by climate change, especially in largest aquaculture producer nations(Reid et al., 2019). Low-temperature stress poses significant challenges to aquaculture production, often resulting in substantial economic losses. For example, a severe cold event in 2010 caused massive fish mortality in the Gulf of Mexico, with significant losses in fish population(Pirhalla et al., 2015). These events show the vulnerability of aquaculture species to extreme temperature fluctuation s(Reid et al., 2022). In China, which is the world's largest aquaculture producer, seasonal cold waves during winter continue to threaten the health and survival of farmed fish(Chu et al., 2024). Therefore, in the context of climate change, we urgently need to understand how fish cope with low-temperature stress.

Oxidative stress and hormonal dysregulation are prominent physiological responses induced by low-temperature stress(Barton, 2002).

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Cold exposure will cause oxidative damage to cellular components such as proteins, lipids, and DNA through increasing the level of reactive oxygen species (ROS), which can subsequently lead to liver damage. Various fish species, including the orange-spotted groupers (*Epinephelus coioides*)(Sun et al., 2019b), zebrafish (*Danio rerio*)(Wu et al., 2015) and Nile tilapia (*Oreochromis niloticus*)(Zhou et al., 2019), have been known to exhibit this phenomenon under cold stress. To counter this, fish have activated their antioxidant defense systems. Specifically, upregulating the levels of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) to reduce the oxidative damage (Wang et al., 2020a). Liver damage caused by cold exposure is also depicted by increasing levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)(Refaey et al., 2023). Low temperatures also impact hormonal regulation, including the production of cortisol and thyroid hormones. A crucial stress hormone is cortisol. It is released to control immune response and metabolism(Sadoul and Geffroy, 2019). However, a long period of cold exposure can remain cortisol levels high. High cortisol levels may impair immune function and increase the risk of infections(Alzaid et al., 2015; Panase et al., 2018). On the contrary, thyroid hormone is released less due to cold stress(Little and Seebacher, 2014). This lowers the metabolic rate and ultimately affects fish growth.

The physiological disturbances caused by low-temperature stress finally reflect deeper molecular alterations that govern these responses. Some important physiological responses, such as metabolism, immune regulation, and cellular homeostasis, are largely influenced by changes in gene expression. The liver is a good target for studying the molecular mechanisms of cold stress in fish, as it plays important roles in both metabolic and immune regulation (Bruslé and Gonzàlez i Anadón, 2017).

In recent years, transcriptomic studies have been widely applied to uncover the molecular mechanisms underlying various environmental stressors in fish, such as hypoxia(Liang et al., 2022), alkalinity(Zhang et al., 2023) and thermal stress(Liu et al., 2025). These studies have revealed complex gene regulatory networks involved in energy metabolism and oxidative stress. Among these, cold stress is other important environmental stressor gained particular attention in aquaculture. For example, in tiger puffer (*Takifugu rubripes*), RNA-seq revealed DEGs involved in metabolism-related pathways under low-temperature exposure(Liu et al., 2022). In largemouth bass (*Micropterus salmoides*), immune and metabolic genes were shown to respond significantly to cold stress(Yu et al., 2023). Similarly, in mrigal carp (*Cirrhinus mrigala*, Hamilton), transcriptomic analysis showed the activation of metabolism and immune pathways, including the AMPK signaling pathway, which indicated increased energy demands during cold exposure(Su et al., 2024). In addition, the NRF2-medicated oxidative stress responses has also been reported as a key regulatory pathway under cold stress in zebrafish(Peng et al., 2022). These studies have revealed the involvement of several signaling pathways, including those related to immune response, oxidative stress, cell survival, and apoptosis. Despite these advances, the integration of physiological and transcriptomic data to clarify cold tolerance mechanisms in spotted sea bass is still limited. By studying the liver transcriptome, we can discovery hub DEGs and pathways that involved in the adaptive response of spotted sea bass to low-temperature stress, which help us to understand the mechanisms of cold exposure tolerance.

Spotted sea bass (*Lateolabrax maculatus*), a eurythermal fish species with a 14 °C to 25 °C ideal temperature variety, is cultivated extensively in coastal areas of China(Chen et al., 2023). Due to its delicious flavors, spotted sea bass is greatly favored by consumers and, therefore, has substantial market demand. However, its sensitivity to temperature fluctuations, particularly during winter, poses a significant threat to production. As water temperatures drop, disruptions in homeostasis may impair growth, weaken immune defenses, and lead to death in extreme cases. Therefore, it is crucial to improve aquaculture practices and maintain sustainable production to fully understand the biological and molecular mechanisms that govern the response of spotted sea bass to low-temperature stress.

The physiological and molecular responses of spotted sea bass to low-temperature stress are investigated in this study. it is essential to investigate how this species responds to cold exposure at both the physiological and molecular levels. The ability to identify biomarkers of cold stress and the pathways involved in temperature regulation will not only contribute to the basic understanding of fish biology but also provide practical applications for improving aquaculture management. For example, selecting cold-resistant strains of spotted sea bass or developing feeding strategies to mitigate cold-induced stress could improve survival rates and production efficiency. Additionally, by understanding the mechanisms underlying cold stress tolerance, we can better predict how spotted sea bass will respond to future changes in climate, including more frequent and severe cold events due to climate change.

2. Materials and methods

2.1. Experimental fish

All fish experiments in this study were approved by the Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201). No endangered or protected species were involved in this experiment.

The experimental subjects were one-year-old spotted sea bass with an average body length of 22.70 ± 1.34 cm and body weight of 103.97 ± 3.85 g), selected from a single batch of artificially cultured fish from Shuangying Aquatic Seeding Co., Ltd., (Dongying, Shandong), which were of uniform size and in good health. A total of 90 fish were randomly assigned to three parallel cages, with 30 fish per cage. The fish were acclimated for 2 weeks under the following conditions: temperature 15 ± 1 °C, salinity 25.67 ± 0.47, dissolved oxygen 7.0 ± 0.5 mg/L, and pH 8.38 ± 0.12. The fish were fed twice daily with commercial marine fish feed (Zhuhai Haiwei Feed Co., Ltd.), and feeding was suspended 24 h prior to the initiation of the experiment. Water was changed every two days, with 50 % of the total water volume replaced.

2.2. Low-temperature challenge and sampling

Prior to the experiment, the water temperature in all three replicate cages was maintained at 15 ± 1 °C. During the experiment, the temperature was gradually decreased at a rate of 1 °C per hour until it reached the target temperature of 5 °C, which was maintained for the remainder of the experiment. Samples were collected at the following time points: 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h under low-temperature stress (5 °C), as well as from a control group maintained at 15 °C (non-stressed). 5 °C was selected as the low-temperature treatment based on the both preliminary trials and previous reports suggesting that temperatures around 4–5 °C induce strong stress responses in spotted sea bass while remaining sublethal(Wang et al., 2022). At each time point, 9 fish (3 randomly selected from each of the three replicate cages) were sampled. The fish were first anesthetized in seawater containing MS-222 (200 mg/L) for sampling. Blood was then drawn and immediately stored at 4 °C for 12 h before centrifugation (4 °C, 5000 rpm, 10 min). The supernatant was preserved and stored at −80 °C for further analysis. After that, liver tissues were sampled and immediately stored at −80 °C for long-term preservation until further use.

2.3. Serum biochemical, hormonal, and antioxidant enzyme assays

We evaluated the effect on low-temperature on spotted sea bass by detecting serum biochemical parameters using a BS-1800 automatic biochemical analyzer with respective reagent kits (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.). Seven serum biochemical indicators were determined, including glucose (GLU), total protein (TP), total cholesterol (TC), ALT, AST, lactate dehydrogenase (LDH), and alkaline phosphatase (ALP). Serum levels of cortisol (Cor) and free thyroxine

(FT4) were measured using 125I-labeled radioimmunoassay (RIA). The liquid-phase equilibrium competitive method was employed for cortisol measurement, while the homogeneous competitive method was used for FT4. Detailed procedures were followed as outlined in the reagent kit instructions, which were obtained from Beijing North Bio-Technology Research Institute Co., Ltd. Additionally, the activities of SOD and CAT in serum were also determined by the xanthine oxidase method and the ammonium molybdate method, respectively. The experimental protocols for these assays were carried out as per the instructions provided with the reagent kits (Nanjing Jiancheng Bioengineering Institute).

2.4. RNA extraction and sequencing

Total RNA from four experimental groups: control (15 °C), 6 h, 12 h, and 24 h post-low-temperature exposure (5 °C) were isolated from liver samples using SparkZol Reagent (SparkJade, Shangdong) following the manufacturer's instructions. RNA concentration, purity and quality were measured via Biodropsis BD-1000 (Beijing, China) and RNAase free agarose gel electrophoresis. To minimize individual variation, equal amounts of RNA from three samples at each time point were pooled. A total of 12 mRNA sequencing libraries were constructed and sequenced using the NovaSeq 6000 platform (illumine) at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

2.5. Data process and differential expression analysis

Quality control of the raw sequencing data was performed using FastQC (version 0.11.9), followed by the removal of low-quality reads and adapter sequences with Trimmomatic (version 0.39) (Bolger et al., 2014). The cleaned reads were aligned to the spotted sea bass reference genome (NCBI BioProject Number: PRJNA408177) using Hisat2 (version 2.2.1)(Kim et al., 2015). Gene expression levels were quantified using featureCounts (version 2.0.1)(Liao et al., 2014), and differential gene expression analysis was conducted with DESeq2 (version 1.30.1) (Love et al., 2014). Differentially expressed genes (DEGs) were defined as those with an adjusted $p < 0.05$ and a $|\log2FoldChange| \geq 1$.

2.6. Time-series analysis and protein-protein interaction (PPI) network construction

Time-series gene expression patterns were analyzed using the Mfuzz package (v2.48.0) in R(Kumar and Futschik, 2007), applying the fuzzy c-means algorithm for soft clustering of the time-series data (FPKM values). The clustering parameters were centers = 6 and fuzzifier (min) = 2.501, to identify distinct clusters based on gene expression trends over time. For PPI networks construction, DEGs within clusters exhibiting similar expression patterns were selected using the online tool STRING (v11.0) (https://www.stringdb.org/), with a confidence threshold of 0.4 to filter interactions. The resulting networks were visualized and further analyzed using Cytoscape (v3.9.1) (http://www.cytoscape.org/), and hub genes were identified using the cytoHubba plugin with the MCC algorithm to assess node centrality(Chin et al., 2014). To identified the biological functions associated with DEGs in each cluster, KEGG pathway enrichment analysis was conducted using the online platform KOBAS(Bu et al., 2021). Pathways with an adjusted $p < 0.05$ were considered significantly enriched, and results were visualized using the ggplot2 package in R(Wickham, 2011).

2.7. Alternative splicing analysis

Differential alternative splicing (DAS) events were quantified using rMATS (v4.01) software (http://rnaseq-mats.sourceforge.net/) to compare RNA-seq data from treatment groups (6 h, 12 h, and 24 h) and the control group. The likelihood-ratio test was applied to compute *p*-values representing the differences in Inclusion Level (IncLevel) between the two groups. To control for multiple comparisons, *p*-values were then adjusted using the Benjamini-Hochberg algorithm to calculate the false discovery rate (FDR). DAS events with an FDR < 0.05 were considered significantly different. KEGG pathway enrichment analysis was also conducted to identified the potential biological functions of DAS genes.

2.8. RNA-seq data validation

To validate the RNA-Seq results, nine DEGs were selected randomly for RT-qPCR analysis. Gene-specific primers as shown in Table S1 were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each experiment was conducted in triplicate, and all reactions were performed using a StepOne Plus Real-Time PCR system (Applied Biosystems). The relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen, 2001). Data visualization was carried out using Graph Prism 8.

3. Results

3.1. Serum biochemical responses under low-temperature stress

As the water temperature reached 5 °C, significant differences in serum biochemical parameters were observed ($p < 0.05$). The GLU concentration increased significantly during the initial stage of low-temperature stress, reaching a peak at 12 h (17.12 ± 0.45 mmol/L). Subsequently, it exhibited a time-dependent decline, and at 72 h, the GLU concentration remained significantly higher than that of the control group (Fig. 1A). The TP and TC concentration both decreased significantly during the early stages of stress and then gradually increased, reaching its lowest level at 6 h (20.52 ± 0.67 g/L) and at 0 h (2.46 ± 0.04 mmol/L), respectively. But the both concentrations at 72 h were still significantly lower than those in the control groups (Fig. 1B, C).

All of the ALT, AST and LDH activity exhibited significant increase followed by a decrease during low-temperature stress, reaching their peak value at 6 h (9.51 ± 0.40 U/L), 12 h (152.22 ± 3.47 U/L) and 6 h (723.42 ± 49.19 U/L), respectively. Overall, all of the activity of experimental groups were higher than that of the control groups except the LDH activity at 48 h and 72 h (Fig. 1D, E, G). By contrast, the serum alkaline phosphatase (ALP) activity showed a significant decreasing trend during the cold stress period, reaching its lowest value at 12 h (25.14 ± 1.66 U/L). Although ALP activity slightly increased at 48 h, it was still significantly lower than that in the control group at 72 h (Fig. 1F).

3.2. Serum hormone levels and antioxidant enzyme activities under low-temperature stress

Cor concentration exhibited a significant increase followed by a gradual decline during cold stress. It peaked at 0 h (171.66 ± 5.89 ng/mL) and then gradually decreased from 0 h to 24 h. A significant reduction was observed at 48 h, with no notable difference between 48 h and 72 h. However, at 72 h, the Cor level was still significantly higher than that in the control group (Fig. 2A). FT4 concentration showed a clear downward trend during the early stages of cold stress, reaching its lowest level at 0 h (1.19 ± 0.04 fmol/mL), followed by a time-dependent increase. The FT4 level at 72 h was still significantly lower than that in the control group (Fig. 2B).

SOD activity significantly increased at the early stage of stress, peaking at 0 h (226.33 ± 2.03 U/mL). It then gradually decreased over time, with a slight increase at 72 h compared to 48 h. However, the SOD activity at 48 h remained significantly higher than that in the control group (Fig. 2C). CAT activity exhibited a significant increase followed by a decline, peaking at 6 h (97.87 ± 1.21 U/mL). Afterward, CAT activity decreased significantly, returning to the pre-stress level at 72 h (Fig. 2D).

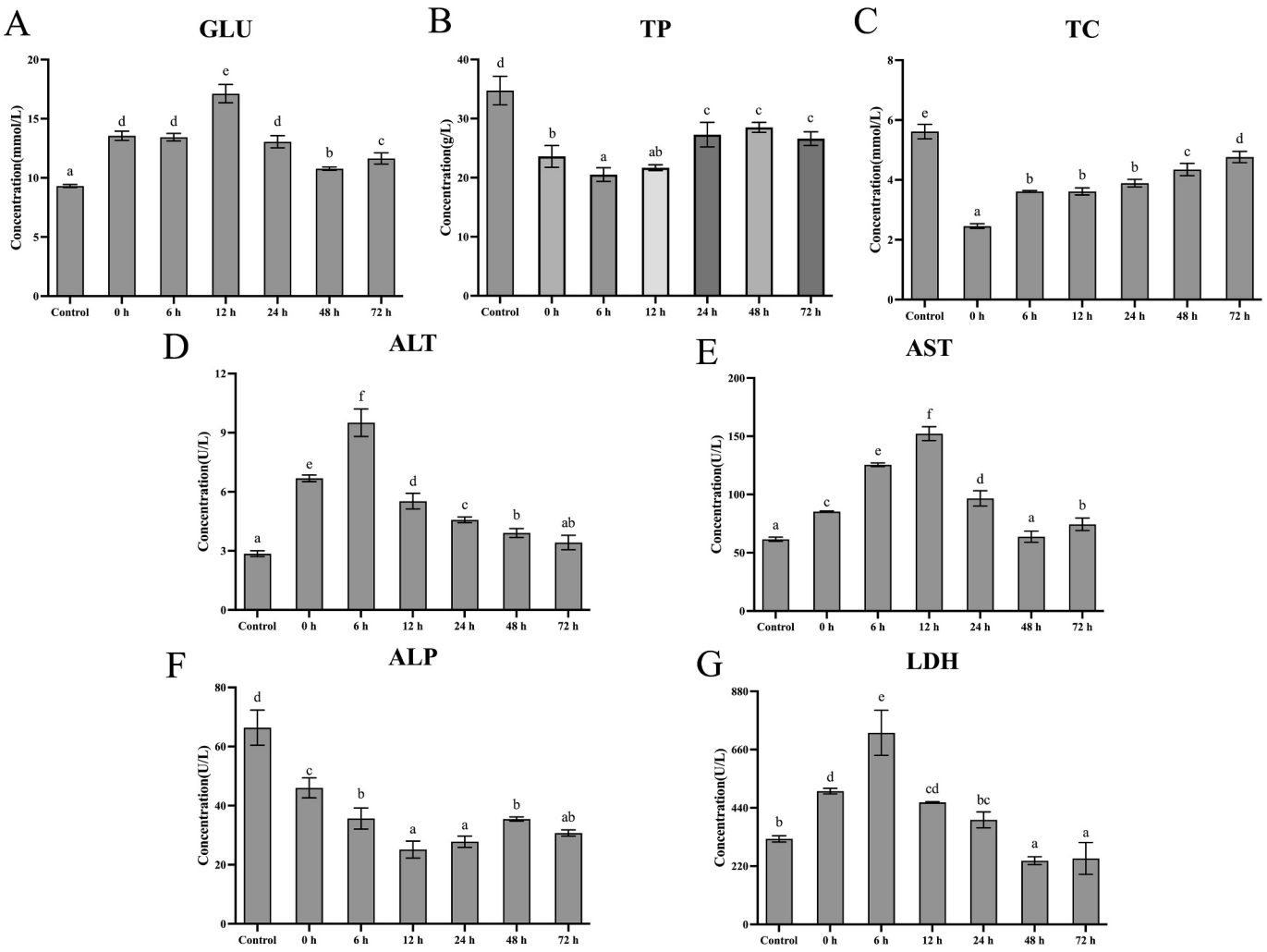

Fig. 1. Serum biochemistry parameters of spotted sea bass under low-temperature stress. (A) glucose (GLU), (B) total protein (TP), (C) total cholesterol (TC), (D) alanine aminotransferase (ALT), (E) aspartate aminotransferase (AST), (F) alkaline phosphatase (ALP) and (G) lactate dehydrogenase (LDH). Data were analyzed by one-way ANOVA followed by Duncan's multiple range test. Different letters above the bars indicate significant differences ($p < 0.05$). Data are presented as mean ± SD ($n = 9$).

3.3. Transcriptome sequencing and quality assessment

A total of 274.81 Mb raw reads were obtained from the sequencing of the 12 cDNA libraries, which included liver tissues sampled from four treatment groups and the control group. After filtering out low-quality sequences, 267.94 Mb clean reads were retained, with an average of 6.7 Gb of clean base per sample. Quality assessment of the sequencing data revealed an average Q20 content of 97.13 %, and an average Q30 content of 92.32 %. The GC content of the sequences ranged from 46.93 % to 48.67 %, with an average of 47.83 %. All clean data were aligned to the reference genome of spotted sea bass, achieving an alignment rate between 89.48 % and 93.29 %, with an average of 91.01 %, which confirms the quality and reliability of the sequencing data for subsequent analyses (Table S2).

3.4. Identification and analysis of DEGs

Differential gene expression was analyzed between the treatment groups (6 h, 12 h, and 24 h) and the control group, generating three comparison groups: 6 h vs. Control, 12 h vs. Control and 24 h vs. Control. A total of 5886 DEGs were identified across the three treatment time points. Specifically, the 6 h vs. Control group exhibited 3873 DEGs, including 1409 upregulated and 2464 downregulated genes (Fig. 3A). The 12 h vs. Control group showed 3831 DEGs, with 1605 upregulated and 2226 downregulated genes (Fig. 3B). The 24 h vs. Control group exhibited 3331 DEGs, with 1489 upregulated and 1842 downregulated genes (Fig. 3C). There are 1757 overlapping DEGs between three comparison groups (Fig. 3D).

3.5. Identification and analysis of hub genes

DEGs were classified into six clusters based on their temporal expression patterns. The clusters contained 1582, 870, 1070, 784, 642, and 938 DEGs, respectively. As shown in Fig. 4A, the DEGs in clusters 4 and 5 exhibited a general increase expression, whereas the DEGs in clusters 1 and 3 showed a progressive decline expression over time under stress treatment. Clusters 2 and 6 displayed irregular expression patterns (Fig. S1). To investigate the functional relationships among the DEGs, PPI networks were constructed for clusters 1, 3, 4, and 5 using the STRING database. The hub genes identified for each cluster are as follows: for cluster 1, 15 hub genes were identified, including *acta2*, *prkdc*, *bptf*, *rhof*, *itgb1a*, *itgb5*, *ehhadh*, *igf1rb*, and others (Fig. 4B); and for cluster 3, 10 hub genes were identified, including *smad3a*, *ptpn11a*, *gata4*, *kat2b*, *jun*, *mapk7*, and others (Fig. 4C); for cluster 4, 10 hub genes were identified, including *wdr43*, *abce1*, *nop48*, *nop56*, *hspa5*, *ddx5*, *dhx15*, *eprs*, among others (Fig. 4D); for cluster 5, 10 hub genes were

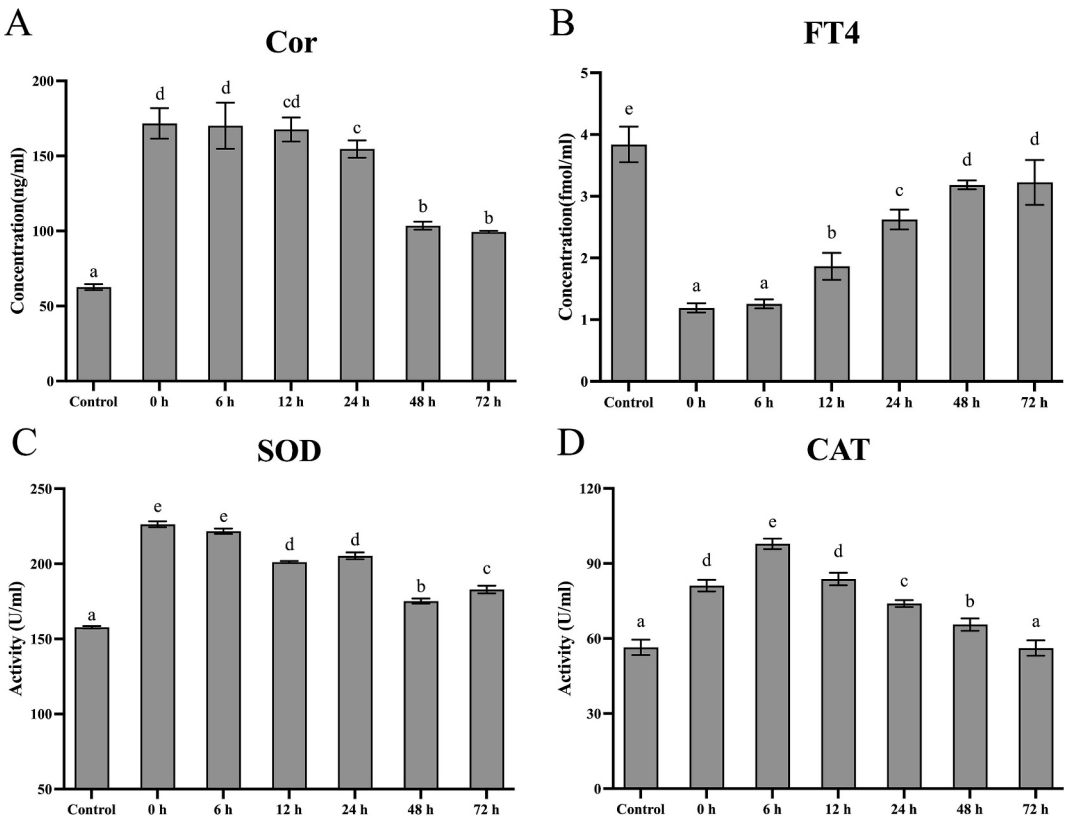

Fig. 2. Serum hormone levels and antioxidant enzyme activities of spotted sea bass under low-temperature stress. (A) cortisol (Cor), (B) free thyroxine (FT4), (C) superoxide dismutase (SOD), (D) catalase (CAT). Data were analyzed by one-way ANOVA followed by Duncan's multiple range test. Different letters above the bars indicate significant differences ($p < 0.05$).

identified, including *hsp90b1*, *hsp90aa1.2*, *ctnnb1*, *pik3ca*, *actc1b*, *pik3r1*, and others (Fig. 4D). All the hub genes in these four clusters were listed in Table S3.

3.6. Pathway enrichment analysis of DEGs

KEGG pathway enrichment analysis was conducted on the DEGs from four clusters to further investigate the biological functions of those genes involved in the low-temperature stress response. For the clusters exhibiting significant upregulation (cluster 4 and cluster 5), DEGs in cluster 4 were significantly enriched in several pathways, including Ferroptosis, FoxO signaling pathway, Spliceosome and some metabolism-related pathways (Fig. 5C, D). Additionally, cluster 5 showed enrichment in immune-related pathways, including NOD-like receptor signaling pathway, as well as cellular process-related pathways, including p53 signaling pathway, Apoptosis, and Autophagy. In contrast, for the clusters exhibiting significant downregulation (cluster 1 and cluster 3), different sets of pathways were enriched. Cluster 1 was significantly enriched in cell adhesion-related pathways, including Focal adhesion, Tight junction, and ECM-receptor interaction, and immune-related pathways, such as RIG-I-like receptor signaling pathway. Meanwhile, cluster 3 showed enrichment in pathways related to genetic information processing, such as Ubiquitin-mediated proteolysis, as well as pathways related to cellular processes, including Autophagy (Fig. 5A, B). Key DEGs in representative pathways were displayed in Fig. S2.

3.7. Identification of DAS events and DAS genes

To investigate the role of alternative splicing in the responses of spotted sea bass to low-temperature stress, DAS analysis was performed using rMATS. A total of 1790 DAS events were observed, primarily consisting of exon skipping (ES) and mutually exclusive exon (ME) events (Fig. 6A). Among them, ES events were the most predominant, accounting for 92.23 % of all DAS events, while ME events were relatively rare, comprising only 7.77 %. The DAS events progressively increased with extended exposure to stress. In total, 414 genes were overlapped between DAS genes and DEGs (Fig. 6B). KEGG pathway enrichment analysis of the genes overlapping genes revealed significant enrichment in RIG-I-like receptor signaling pathway, NOD-like receptor signaling pathway, and glutathione metabolism pathway (Fig. 6C).

3.8. Validation of RNA-seq data by RT-qPCR

To confirm the RNA-seq results, RT-qPCR was performed on nine randomly selected DEGs. The expression patterns observed were consistent with those form RNA-seq, with $R^2 = 0.946$ (Fig. S2). These results validate the RNA-seq data, supporting the accuracy and reliability of the transcriptomic analysis (Fig. 7).

4. Discussion

Low-temperature stress represents a significant environmental challenge for fish species, impacting their growth, immunity, and overall health(Barton, 2002). Spotted sea bass is an economically important species in China, but its sensitivity to temperature fluctuations limits aquaculture expansion and efficiency during colder months. Liver is an essential organ for respondence of low-temperature stress in fish to maintain metabolism and immune homeostasis. Understanding the physiological and molecular responses of spotted sea bass to low-temperature stress is critical for optimizing breeding programs and improving aquaculture management practices to ensure the sustainability of the industry. In the present study, we investigated the physiological and transcriptomic alterations in spotted sea bass under low-temperature conditions, focusing on key biomarkers and gene

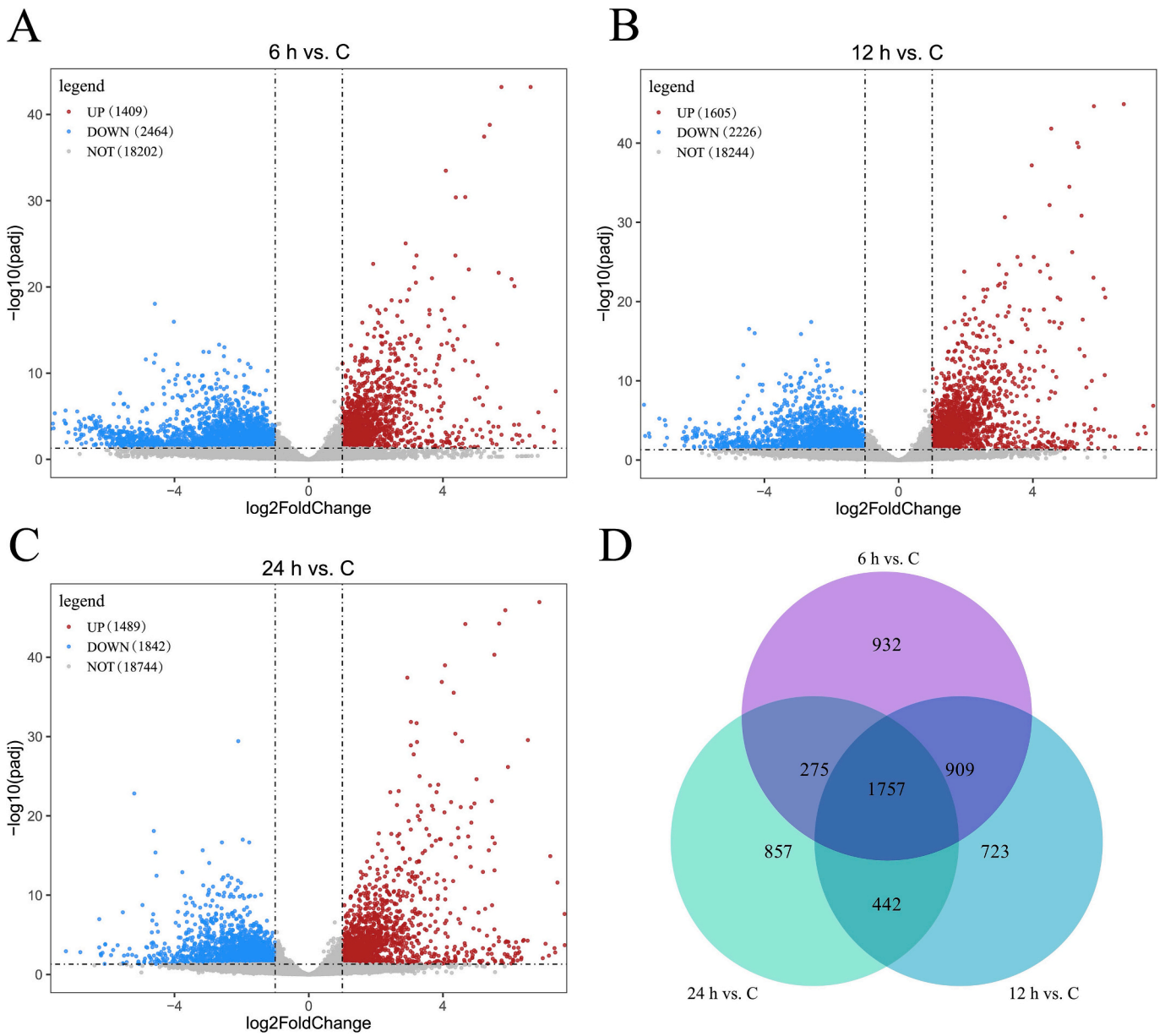

Fig. 3. Identification of differentially expressed genes (DEGs) in the liver of spotted sea bass under low-temperature stress. Volcano plots of DEGs in (A) 6 h vs. Control, (B) 12 h vs. Control and (C) 24 h vs. Control. Red dots represent up-regulated DEGs, blue dots represent down-regulated DEGs and grey dots represent genes that are not differentially expressed. (D) Venn diagram showing the overlap of DEGs among the three comparison groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression patterns to uncover the mechanisms involved in cold stress adaptation (Fig. 8).

4.1. Physiological and biochemical responses to low-temperature stress

Serum biochemical parameters, such as GLU, TP, TC, ALT, AST, ALP, and LDH, have long been used to assess the physiological condition under stress responses in fish. In our study, significant fluctuations in these markers were observed at 6 h, 12 h, 24 h, and 72 h after low-temperature stress exposure. Serum GLU, TP and TC are critical indicators of metabolic responses to stress in fish, while GLU, as a sources of ATP, is more sensitive to temperature changes than TP and TC (Li et al., 2020). In this study, GLU levels significantly increased and peaked at 12 h, indicating an adaptive metabolic response was activated to mobilize energy reserves and cope with cold stress in spotted sea bass. This result is consistent with the general physiological response to cold stress in fish species(Inoue et al., 2008). Although the GLU level returned to baseline at 24 h, a secondary increase at 72 h may reflect prolonged stress or continued metabolic demand in response to the low temperature. Additionally, serum TP and TC, both synthesized in the liver, exhibited significant decline at the beginning of cold stress. These declines suggest disruptions in proteins synthesis and overall metabolic functions under cold exposure(Refaey et al., 2022). Similar trend of TP and TC were observed on other species under cold stress, such as such as cobia (*Rachycentron canadum*) and large yellow croaker (*Pseudosciaena crocea*)(Ji et al., 2009; Yu et al., 2022). Furthermore, metabolic pathway was significantly enriched, and several key metabolic genes, such as *pfkm*, *acsl4* and *glud1*, exhibited significant differential expression during cold exposure (Fig. S3). These results further illustrated the important involvement of metabolism in energy production under cold stress response. Additionally, ALT and AST are key markers of liver function, and their increased activity suggests liver cell damage and changes in

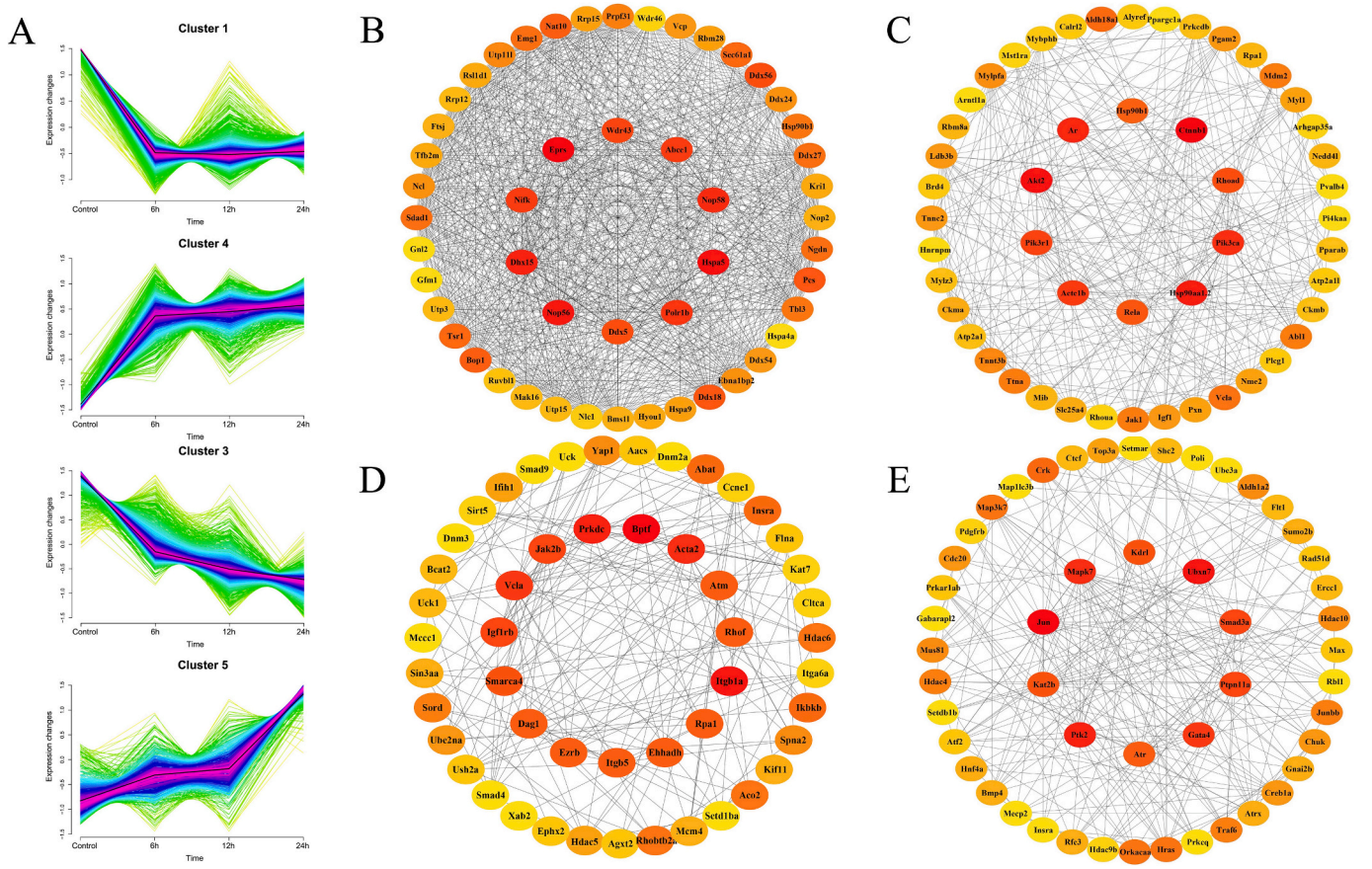

Fig. 4. Clustering of DEGs based on time-series analysis and protein-protein interaction (PPI) networks. (A) Dynamic expression patterns of four selected clusters: cluster 1 and cluster 3 show an upward trend, while cluster 4 and cluster 5 show a downward trend. Different colors represent the match degrees between gene expression changes and the major cluster trends. Fuchsia, blue and green indicate high, moderate and low match degrees, respectively. PPI networks of DEGs in (B) clusters 1, (C) clusters 3, (D) clusters 4 and (E) clusters 5. Nodes represent proteins encoded by DEGs, and edges represent predicted or known interactions. The color gradient of nodes reflects their degree of connectivity, with redder nodes indicating higher degrees. Nodes in the central circle of the network with darker red color are defined as hub genes, indicating key regulators with high degrees of interaction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell membrane permeability, which are also biomarkers of stress (Dahlhoff, 2004). In this study, significant increases in serum ALT and AST activities were observed at the early stages of cold stress, which are consistent with the trend in researches of turbot (*Scophthalmus maximus*) (Ji LiQin et al., 2016), Nile tilapia(Yilmaz et al., 2021) and pallas (*Acipenser stellatus*)(Shahsavani et al., 2010). LDH is another important enzyme involved in energy metabolism(Atli et al., 2015). In other aquatic species, its levels have been reported to increase under stress conditions(Sun et al., 2019a; Xiaolong et al., 2018). Consistent with these findings, LDH in our study exhibited a similar increase trend as ALT and AST, reflecting that low-temperature stress may have caused liver damage in spotted sea bass. The elevated LDH levels may also indicate an increased energy demand to cope with the effects of cold exposure. In our study, ALP showed a significant decrease during the low-temperature stress, which suggesting possible liver damage, metabolic dysfunction, and immune mechanisms impaired(Cheng et al., 2017; Lan et al., 1995). In pufferfish (*Takifugu obscurus*), ALP was also found that has similar trend under stress (Cheng et al., 2017). These results not only suggested the metabolism alterations but also indicated the liver damaged in spotted sea bass under cold stress.

4.2. Antioxidant defense and hormone response to low-temperature stress

Cor and FT4 are critical hormones involved in regulating stress and metabolic in fish(Leonard et al., 2014). In this study, we observed a significant increase in Cor levels, indicating the spotted sea bass was under stress. A study in Nile tilapia(*O. mossambicus*) reported a significant increase of Cor level at 24 h post low-temperature exposure (Yilmaz et al., 2021). Similarly, in this study, the Cor level exhibited significantly increase at all sampling time points, suggesting a more rapid and sustained endocrine stress response. Such prolonged elevation of Cor may suppress immune function and increase the susceptibility to pathogen infections in spotted sea bass. Conversely, the concentration of FT4 reach at lowest level at 0 h of cold stress followed by a time-dependent increase. This indicates thyroid hormone synthesis was suppressed under cold stress, which may contribute to reduce metabolic activity and growth in spotted sea bass during prolonged exposure to low temperatures. The increase of Cor and the decrease of FT4 was also observed in other aquatic species(Hunt et al., 2012).

Low temperatures can significantly increase the levels of ROS in fish cells, which could lead to severe cellular damage. To counteract this, antioxidant enzymes such as SOD and CAT will participate in regulating ROS and repairing oxidative damage(Nordberg and Arnér, 2001). Studies in zebrafish have demonstrated that the levels of ROS, SOD and CAT mRNA were increased after low-temperature exposure(Tseng et al., 2011; Wu et al., 2015). In the present study, we also found that a significant increase in both SOD and CAT activities during the early stages of cold stress. These results indicated that the antioxidant defense mechanisms might be activated to protect against oxidative damage in spotted sea bass under cold stress. We could also found the increased levels of SOD and CAT in the studies on discus fish (*Symphysodon aequifasciatus*)(Wen et al., 2018) and crucian carp (*Carassius auratus*)

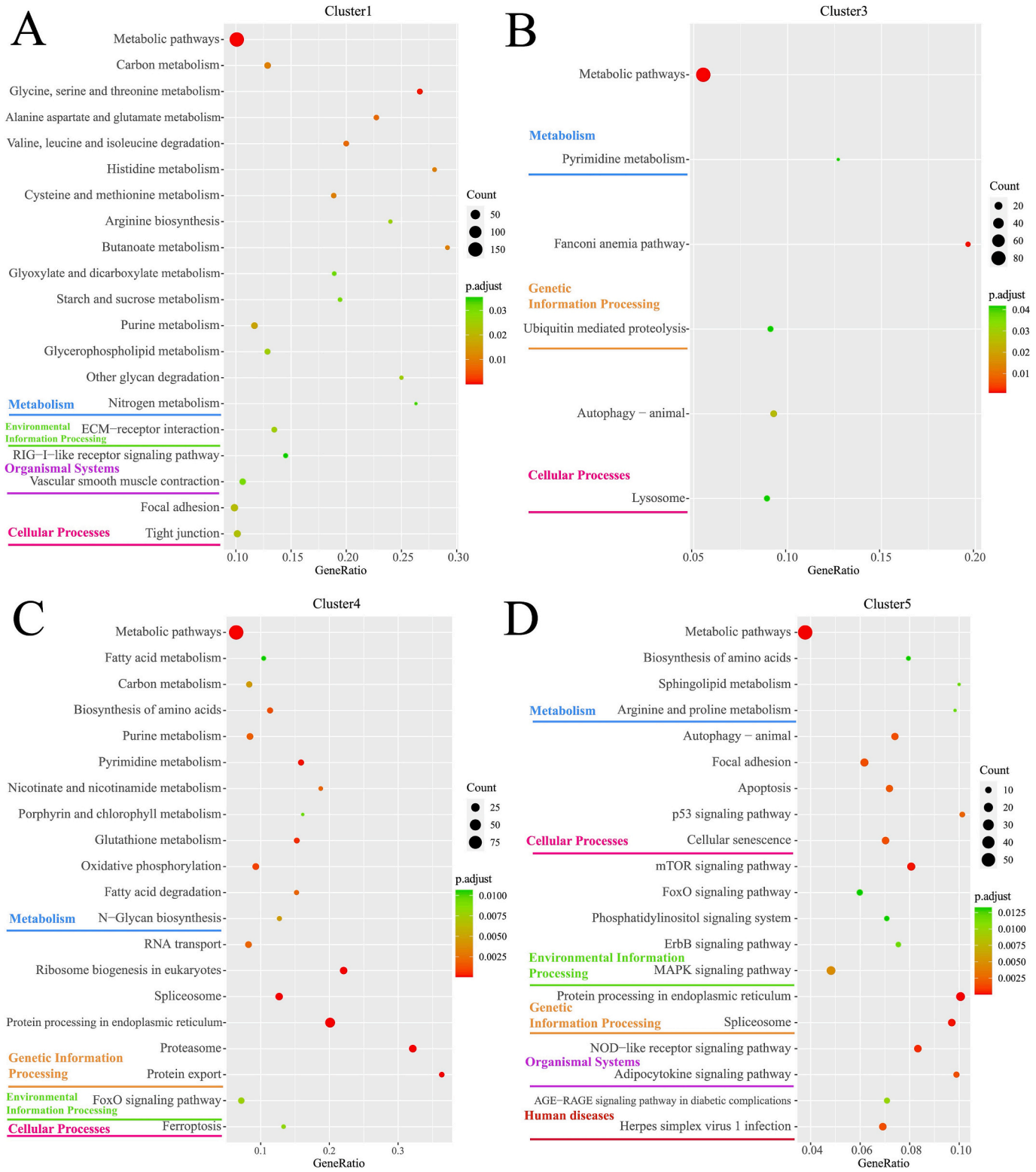

Fig. 5. KEGG enrichment analysis of DEGs in clusters with differential expression patterns. Significantly enriched pathways (adjusted $p < 0.05$) identified in (A) cluster 1, (B) cluster 3, (C) cluster 4 and (D) cluster 5. The color of the dots represents the adjusted p and the size represents the number of genes enriched in each pathway.

(Tang et al., 2023). However, after 6 h of exposure, both SOD and CAT activities showed a decreasing trend and CAT activities returned to baseline levels by 72 h. This suggests that prolonged exposure to cold stress may impair the synthesis of antioxidant enzymes and reduce the ability of spotted sea bass to counteract ROS.

4.3. Transcriptomic changes in response to cold stress

To further understand the molecular mechanisms of physiological responses of spotted sea bass under low-temperature stress, we conducted transcriptomic analysis on the liver at the acute phase of cold

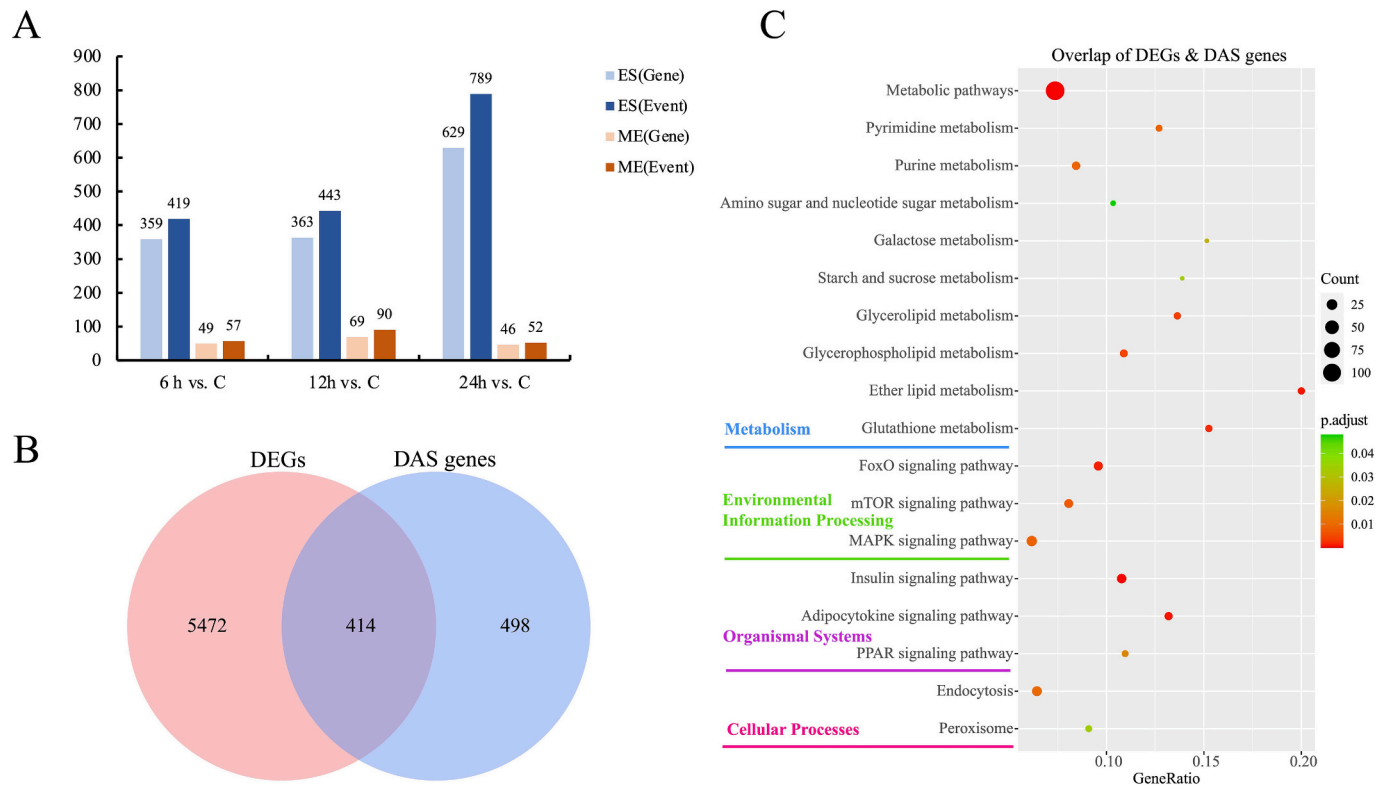

Fig. 6. Identification and functional analysis of differential alternative splicing (DAS) events and genes in the liver of spotted sea bass under low-temperature stress. (A) Numbers of DAS events and genes of exon skipping (ES) and mutually exclusive (ME) in the three comparison groups. (B) Venn diagram showing the overlap between DAS genes and DEGs. (C) KEGG enrichment analysis of the overlapping DAS genes and DEGs (adjusted $p < 0.05$). The color of the dots represents the adjusted p and the size represents the number of genes enriched in each pathway.

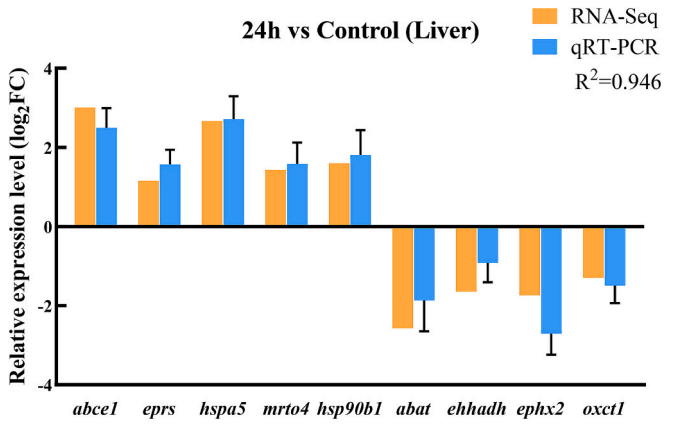

Fig. 7. qPCR validation of DEGs in liver of spotted sea bass under low-temperature stress ($n = 3$).

stress, which is at 0, 6, 12, and 24 h after exposure to 5 °C. This time frame was selected based on our preliminary results, which showed that key serum biochemical parameters exhibited their most dramatic changes within the first 24 h of cold exposure. These findings suggest that the early stage represents the most active period of physiological regulation and stress response. Therefore, we prioritized capturing this acute phase to better understand the dynamic molecular and physiological adjustments that occur immediately after the onset of cold stress. Our study revealed a set of DEGs that were significantly enriched in metabolism, immune response and cell stress response and death. These results offer deeper insights into how fish cope with the physical damage caused by cold stress.

Cold exposure impaired both innate and acquired immune responses in fish(Abram et al., 2017). Although the activity of both decreases with low temperature, they still function and compensate for each other's inhibition. This allows fish to maintain a certain degree of immune defense under cold stress(Nikoskelainen et al., 2004). For instance, at lower temperature, the response of rainbow trout (*Oncorhynchus mykiss*) to bacterial infections becomes slower and weaker(Raida and Buchmann, 2007). Moreover, in Nile tilapia, lower temperature lead to decreased survival rates during vaccination (Wang et al., 2020b). Our research found that downregulated genes in Cluster 1 were significantly enriched in the RIG-I-like receptor signaling pathway, which suggests low-temperature exposure may weaken the immune defenses of spotted sea bass, potentially impairing their ability to defend against to pathogens. In addition, we also found that the overlapping genes between DEGs and DAS genes were significantly enriched in immune-related pathways, such as RIG-I-like receptor signaling pathway and NOD-like receptor signaling pathway, indicating that alternative splicing events may participate in the stress responses of spotted sea bass under cold exposure.

Furthermore, we observed the upregulated of the p53 signaling pathway and the ferroptosis pathway in this study, both of which are important for cellular stress responses. It is known that the p53 signaling pathway can regulate cell cycle arrest, DNA repair, and apoptosis. P53 activation can prevent the proliferation of damaged cells, and initiate apoptosis in severely damaged cells. This process may protect the tissues from further damage(Maddocks et al., 2013; Yuan et al., 2017). Therefore, we infer that it may activate these cellular defense mechanisms in spotted sea bass to cope with cold-induced cellular damage, particularly in liver(Kastenhuber and Lowe, 2017). The p53 signaling pathway and p53 gene were also identified in pompano (*Trachinotus ovatus*) and large yellow croaker (*Larimichthys crocea*) under cold stress (Qian et al., 2020; Zhang et al., 2024). Ferroptosis is a regulated form of cell death characterized by iron-dependent lipid peroxidation, which can be triggered

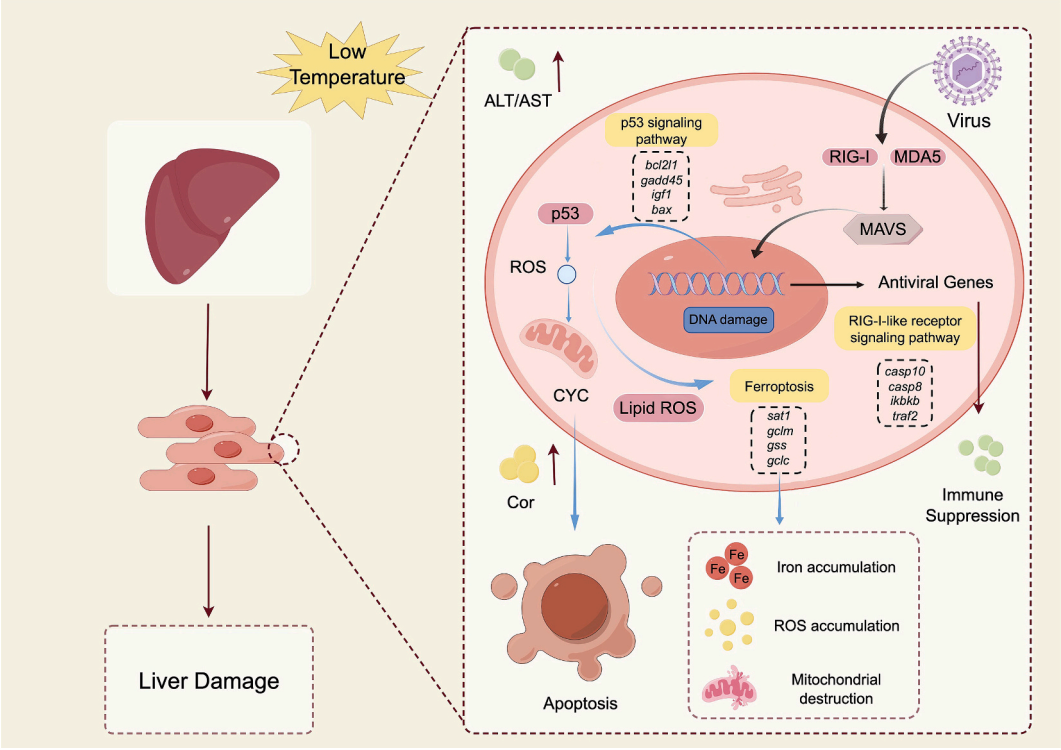

Fig. 8. Schematic regulatory network of low-temperature stress response in the liver of spotted sea bass using Figdraw (www.figdraw.com). The left panel shows the overall impact of low temperature on liver function, leading to elevated ALT, AST and Cor levels. The right panel illustrates the detailed cellular mechanisms: low-temperature stress activates the p53 signaling and ferroptosis, resulting in apoptosis, iron accumulation, ROS generation, and mitochondrial destruction. The suppression of the RIG-I-like receptor signaling pathway induces immune suppression to antiviral responses. DEGs involved in pathways are shown along the pathway in black dashed boxes. Pathways significantly enriched in DEGs (adjusted $p < 0.05$) are highlighted in yellow boxes. Detailed information on genes involved in these pathways are shown in Table S4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by oxidative stress(Endale et al., 2023). Given that cold exposure is known to elevate ROS production. The excessive accumulation of ROS can initiate ferroptosis, which in turn results in liver damage(Huang et al., 2023). In our study, several key genes related to glutathione metabolism and lipid peroxidation, such as *sat1*, *acsl1*, *gss*, *gclc* and *gclm*, were significantly upregulated, indicating potential activation of the ferroptosis pathway under low-temperature stress. Previous studies have reported similar findings, such as ferroptosis-induced organ damage under low-temperature in Nile tilapia (Zhou et al., 2019). Therefore, we speculate that ferroptosis might contribute to cellular damage in spotted sea bass under cold stress. The activation of both the p53 signaling and ferroptosis pathways indicates a series of cellular response in spotted sea bass were activated, aimed to reducing oxidative damage, regulating cell survival, and maintaining tissue homeostasis under cold stress. However, long-term activation of these pathways may actually have harmful effects, including excessive cell death, which may also impair the health of fish. Future studies are needed to validate the role of the p53 signaling and ferroptosis pathways in cold stress by integrating physiological assays and further expression analysis of ferroptosis-specific regulators.

In conclusion, our study reveals the dynamic physiological and molecular responses of spotted sea bass to low-temperature stress. By integrating physiological and transcriptomic data, we have identified the physiological adaptations and key pathways in spotted sea bass under low-temperature stress. The findings contribute to our understanding of the mechanisms underlying cold stress tolerance in fish and provide valuable insights for the sustainable management and breeding of aquaculture species in the face of climate change.

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CRediT authorship contribution statement

Yani Dong: Writing – original draft, Visualization, Formal analysis. **Yuan Zheng:** Visualization, Investigation, Formal analysis. **Haishen Wen:** Resources, Funding acquisition, Conceptualization. **Yonghang Zhang:** Software, Methodology. **Xin Qi:** Resources, Conceptualization. **Lingyu Wang:** Resources. **Chong Zhang:** Visualization, Methodology. **Kaiqiang Zhang:** Resources, Methodology. **Shaoshen Yang:** Funding acquisition. **Yun Li:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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.

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Data availability

Data will be made available on request.

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