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Research article

Effect of dissolved oxygen and ammonia nitrogen on *Culter alburnus*: Physiology, biochemistry, and molecular analyses

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ABSTRACT

Culter alburnus (topmouth culter)is an economically valuable freshwater fish. However, its insufficient tolerance to dissolved oxygen (DO) and ammonia nitrogen (AN) hinders its industrialisation. 360 experimental fish (4.87 ± 1.10 g) were placed in breathing chambers (oxygen level was 0.70–6.50 mg/L) or water tanks (control AN, 0 mg/L; low AN, 8 mg/L; high AN, 16 mg/L). This study analysed the effects of DO and AN on *C. alburnus* at physiological, biochemical, and molecular levels. (1) Physiology level: the floating point, coma critical point, and coma point at 20 °C group were significantly higher than those at 30 °C. The oxygen consumption rate of *C. alburnus* at 20 °C, 25 °C, and 30 °C was (256.65 ± 25.87), (470.47 ± 83.84), and (520.87 ± 55.40) mg/kg.h. The LC50 of AN after 96 h was 24.13 mg/L, and the safe concentration was 2.41 mg/L. The survival rate in the high AN group was significantly lower than that in the other two groups. (2) Biochemistry level: The change curves of antioxidant enzyme activity in the liver tissue under hypoxic stress reached a maximum at 12 h and then decreased. In addition, the increase and decrease in enzyme activity (except malondialdehyde) in the high AN group was lower than that in the low AN group. (3) Molecular level: the angiotensin-converting enzyme and carboxypeptidase genes were the major differentially expressed genes (DEGs) in hypoxic stress, and the DEGs were mainly enriched in the ABC transporter signal transduction pathway. In addition, the serum/glucocorticoid-regulated kinase, stearoyl-CoA desaturase, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes were among the major DEGs under high AN stress. The DEGs were mainly enriched in steroid biosynthesis or glycine, serine, and threonine metabolism transporter signal transduction pathways. In summary, it is necessary to focus on the DO and AN during *C. alburnus* breeding.

1. Introduction

Abnormal weather, sudden changes in water quality, and long transportation times result in low levels of dissolved oxygen (DO) and high levels of ammonia nitrogen (AN) during fish cultivation [1–3]. When the intensity of DO or AN stress in water is lower than the tolerance limit of fish, the body can adjust itself to adapt to changes in the external environment. However, excessive stress causes tissue and organ damage, imbalances in environmental homeostasis, and imbalances in the immune system, affecting the survival rate

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[4–6]. Romano et al. and Huang et al. showed that when the low DO or high AN stimulation time was too long or the stimulation intensity exceeded the body regulation threshold, the antioxidant system of the body was damaged, besides, the activity of some antioxidant-related enzymes decreased, resulting in a reduction in the ability to scavenge free radicals and an increase in lipid peroxidation products, thus, damaging the non-specific immune defence system of the body [7,8].

Culter alburnus (topmouth culter) is an economically valuable freshwater fish species in China [9]. *C. alburnus* are widely distributed in various water systems and affiliated lakes in the Yangtze River Basin [10]. However, insufficient tolerance to hypoxia and AN is an obstacle to the industrialisation of *C. alburnus*. Zhu et al. measured the oxygen consumption rate and suffocation point of *C. alburnus* at various temperatures [11]. Hu et al. discussed the effects of transportation time and density on the oxygen consumption rate of *C. alburnus* [12]. Li suggested that with an increase in temperature, the oxidation process in the living cells of the fish is accelerated, the consumption of oxygen is increased, and the role of supplying oxygen to various tissues is also slightly increased, which are the same responses as those observed during hypoxia caused by insufficient oxygen in the environment. The respiratory movement of fish is accelerated by an increase in temperature, which increases oxygen consumption and affects the fish [13]. However, tissue and organ damage as well as oxidative stress during hypoxia have not been intensively investigated. Therefore, to better breed and produce *C. alburnus*, it is important to test its stress resistance and explore organ histological changes and oxidative stress mechanisms under hypoxic conditions to further understand its anti-hypoxic ability. AN is a water quality index that needs to be considered during *C. alburnus* breeding; however, at present, fish are cultured in freshwater, such as *Mylopharyngodon piceus* (black carp) [3] and *Pelteobagrus fulvidraco* (banded catfish) [14], and there have been reports on the mechanism of immunotoxicity under AN stress; however, to our knowledge, there are no relevant reports on Cultrinae subfamily fishes. Therefore, it is necessary to study the changes in antioxidant enzyme activities and the molecular mechanism of the response to AN stress in fish treated with different concentrations of AN for different durations to explore the oxidative stress mechanism of fish in the process of AN stress and to guide production activities.

This study aimed to analyse the effects of low DO and high AN levels on *C. alburnus*, including the evaluated parameters. To guide extreme situations in the breeding process, the physiological response and tolerance of *C. alburnus* to low DO and high AN environments were investigated, which could provide a basis for healthy *C. alburnus* breeding.

2. Materials and methods

2.1. Fish rearing conditions

The experiments were conducted at the Zhejiang Institute of Freshwater Fisheries in Huzhou, China. Experimental fish of similar specifications (4.87 ± 1.10 g) used in the experiment were healthy and were transiently reared under the same conditions. Experimental fish were placed in breathing chambers or water tanks. During the test, feeding was stopped.

2.2. Determination of hypoxia-related indexes at the physiology level

2.2.1. Determination of floating, coma critical, coma, suffocation critical, suffocation, and death points at 20 °C, 25 °C, and 30 °C

For the breathing chamber, a 5 L wide-mouthed bottle sealed with liquid paraffin was used, from which the water sample was sucked by the siphon method [15]. Then, 10 experimental fish were placed in each breathing chamber and nine breathing chambers were used. The floating point, coma critical point, coma point, suffocation critical point, suffocation point, and death point were measured at 20 °C, 25 °C, and 30 °C, respectively (initial DO was 6.5 mg/L). Furthermore, the fish were not fed, and the water was not changed. Three parallel observations were performed for each parameter group.

The DO value of the water (measured with a HQ 30d portable DO instrument) when the first fish floating head appeared was used as the floating point. The DO value when the first fish was observed to be out of balance and lying on its side was used as the coma critical point. The DO value when 50% of the fish population was in a coma was used as the coma point. The DO value when the first fish died was used as the suffocation critical point. The DO value when 50% of the fish population died was used as the suffocation point. The DO value when all fish were dead was used as the death point [11].

2.2.2. Determination of the daily average oxygen consumption rate and diurnal variation of the oxygen consumption rate at 20 °C, 25 °C, and 30 °C

The mouth of the breathing chamber (5 L wide-mouthed bottle) was sealed with a rubber plug, and three holes were drilled in the rubber plug to place the inlet and outlet pipes and the water temperature gauge in these locations, respectively. Then, 10 experimental fish were placed in each breathing chamber at 20 °C, 25 °C, and 30 °C. Water samples were collected every 2 h (from 14:00 until the next day at 12:00) and the average value was calculated. After the experiment, water flow was monitored, and the weights of the fish were measured. The electrochemical probe method was used to measure the DO. The oxygen consumption of the experimental fish using the formula $q = (q' - q'')V$, where q (mg) is the measured oxygen consumption, q' (mg/L) is the oxygen content of the incoming water sample, q'' (mg/L) is the oxygen content of the outflow water sample, and V (L) is the volume of water flowing through the breathing chamber at each time interval. The total oxygen consumption of the experimental fish using formula $Q = q1 + q2 + q3 \ldots + qi$, where Q (mg) is the total oxygen consumption of the experimental fish during the entire experiment, and qi (mg/L) is the ith measured oxygen consumption. The oxygen consumption rate was calculated as $f = Q/24W$, where f [mg/(kg.h)] is the oxygen consumption rate and W (kg) is the body weight of the experimental fish [16,17] (Fig. 1).

2.3. Determination of an stress-related indexes at the physiology level

2.3.1. Determination of the lethal concentration

Conventional acute biological toxicity tests were performed. First, 10 experimental fish of similar specifications were placed in each water tank at ammonia-nitrogen concentrations of 0, 10, 20, and 30 mg/L, respectively, and 12 water tanks were used in total. During the tests, the AN concentration was monitored over time to ensure stability. The water temperature was maintained at 20–22 °C. After 96 h of stress, the survival rate, half-lethal concentration (96 h LC50) and safe concentration (SC) of AN for 96 h were calculated. The 96 h LC50 was 24.13 mg/L, and the SC was 2.41 mg/L.

2.3.2. An stress

Considering the actual production and the 96 h LC50, control (0 mg/L), low concentration (8 mg/L), and high concentration (16 mg/L) groups were used for the test. Overall, 30 experimental fish of similar specifications were placed in each water tank in three parallel groups. The experimental total ammonia-nitrogen concentration was determined using an analytically pure ammonium chloride reagent. During the test, feeding was stopped and the concentration of total AN was monitored every 4 h. The amount of water changed each time was not more than 20%, the water temperature was 20–22 °C, and 24 h continuous micro-aeration was used to ensure that the DO content was more than 7.0 mg/L. The survival rate of each group was calculated after 96 h, and samples were collected for subsequent analysis.

2.4. Hepatic antioxidant assays

Liver samples (three tube samples randomly selected from three fish in each group) were collected at 0, 6, 12, and 24 h under hypoxia stress and at 0, 6, 12, 24, 48, and 96 h under AN stress. The water temperature was 20–22 °C, and 24 h continuous micro-aeration was used to ensure that the DO content was more than 7.0 mg/L under AN stress. The samples were stored in liquid nitrogen and then transferred to a −80 °C ultra-low temperature refrigerator for storage. The tissue homogenate was prepared by grinding the tissue and adding 9 mL of normal saline to each gram of tissue to be tested. The homogenate was then transferred to 2 mL centrifuge tubes and centrifuged at 1006×*g* at 4 °C for 10 min [18]. The supernatant was separated and kept in a 4 °C refrigerator for temporary storage and was later used for measuring superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) activities. The measurement steps were carried out according to the manufacturer's instructions, and the absorbance was measured using a spectrophotometer (Mapada Instruments UV-1200) at the corresponding wavelengths.

2.5. Transcriptome analysis at the molecular level

The liver samples under hypoxic stress were stored in liquid nitrogen (liver samples of three fish sampled at 0 and 24 h, respectively). The liver samples under high concentration (16 mg/L) ammonia-nitrogen stress were stored in liquid nitrogen (liver samples of three fish sampled at 0 and 96 h). Samples were transferred to a −80 °C ultra-low temperature refrigerator for storage before transcriptome sequencing. To obtain high-quality clean data, joint sequences and low-quality reads were removed from the raw data using data filtering. The clean data were sequenced to obtain an UniGene library of the species. The quality of the sequencing library was evaluated using randomness and saturation tests. After library quality evaluation, expression analysis, gene structure analysis, differential expression analysis, functional annotation, and functional enrichment of differentially expressed genes (DEGs) were performed according to the expression number of genes in different samples or different sample groups to explore relevant pathways and key genes.

2.6. Real time PCR validation

Through total RNA extraction of liver samples, RNA concentration determination, cDNA synthesis, and real-time PCR, the sample cDNA obtained by reverse transcription was diluted four times; the internal reference gene, 18s RNA, was selected; and the SYBR

Fig. 1. Determination of hypoxia-related indexes of *C. alburnus*.

reagent method was used to analyse the expression of the obtained samples. Fluorescent 96-well plates were placed in an ABI 7500 real-time PCR apparatus for amplification. Fluorescence quantitative PCR reaction procedure: 95 °C, 30 s; 95 °C, 5 s, and 60 °C, 60 s for 40 cycles, followed by 95 °C for 5 s and 60 °C for 1 min. The dissolution curve was detected by the $2^{-\triangle\triangle C}$ method, the relative expression levels between samples were obtained, and a bar chart was drawn [19].

2.7. Statistical analysis

Results were subjected to analysis of variance (ANOVA) using the SPSS 17.0 computer program and previous arc-sine transformation of data. An ANOVA was performed for each experiment. Tukey's multiple comparison test was used as the post-hoc test. Kaplan Meier was applied for survival and probit program was used for LC50. All data are presented as the mean ± SD. For all statistical tests, P values ≤ 0.05 were considered significant.

3. Results

3.1. Effects of DO and an on C. alburnus at the physiology level

3.1.1. Hypoxia-related indexes under different water temperatures

The DO value basically decreased from the floating point to the death point when the temperature increased from 20 °C to 30 °C. The hypoxia-related indexes of the floating point, coma critical point, and coma point at 20 °C were significantly higher than those at 30 °C ($P < 0.05$) (Fig. 2).

The oxygen consumption rate at 20 °C was significantly lower than that at 30 °C ($P < 0.05$). The results of the average oxygen consumption rate at night and during the day at 20 °C, 25 °C, and 30 °C showed that at 20 °C and 25 °C, the oxygen consumption rate was higher at night (0.237–0.291 mg/g.h at 20 °C and 0.220–0.278 mg/g.h at 25 °C from 18:00–8:00) and lower during the day (0.180–0.224 mg/g.h at 20 °C and 0.193–0.230 mg/g.h at 25 °C from 10:00–16:00). However, the oxygen consumption rate at 30 °C was higher during the day (0.217–0.288 mg/g.h at 30 °C from 18:00–8:00), especially from 8:00–14:00 in the morning (0.254–0.289 mg/g.h) to 18:00–20:00 in the evening (0.283–0.288 mg/g.h), when the oxygen consumption rate increased sharply, reaching the peak value for the day, and then decreased rapidly, with the lowest value of the day at 0:00 at night (0.217 mg/g.h) (Fig. 3).

However, *C. alburnus* swam normally at the beginning of suffocation. A few hours later, with a decrease in the DO in the water, the breathing rate of the fish accelerated, and they became irritated and began to float. The fish body became highly restless when the DO in the water was further reduced. They occasionally swam to the lowest depth of the water, quickly swam to the surface, concentrating on the mouth of the bottle, pressed the bottle cap with their mouth, and even swam around the breathing chamber. After several attempts, it calmed down again. At this time, the body of the fish was unbalanced and inclined towards the water surface, the head was upward, and the stomach was tilted. Subsequently, the fish slowly sank to the bottom of the bottle, their breathing rate became slower, the gill cover opened and closed slightly, the body colour became lighter, and finally, the fish died with spasms.

3.1.2. An acute toxicity stress test

The results showed that the mortality rates of juveniles under high ammonia-nitrogen stress for 96 h at ammonia-nitrogen concentrations of 0, 10, 20, and 30 mg/L (Table 1). The LC50 of AN after 96 h was 24.13 mg/L, and the safe concentration (SC) was 2.41 mg/L calculated using SPSS.

The results of the AN stress analysis showed that the AN concentration and stress time affected the survival rate of *C. alburnus*. The

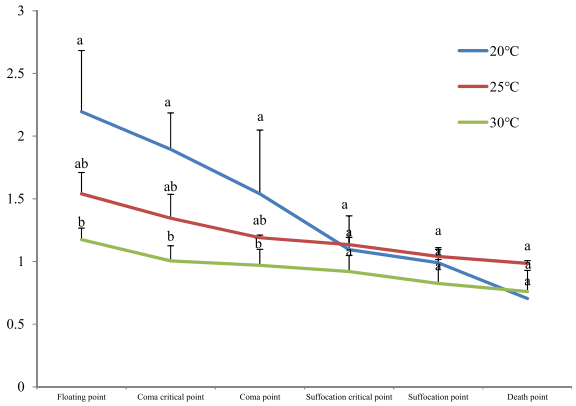

Fig. 2. Determination of the floating point, coma critical point, coma point, suffocation critical point, suffocation point, and death point of *C. alburnus* at 20 °C, 25 °C, and 30 °C. The error bars represent SEM and the columns marked with different letters indicate statistically significant differences ($P < 0.05$).

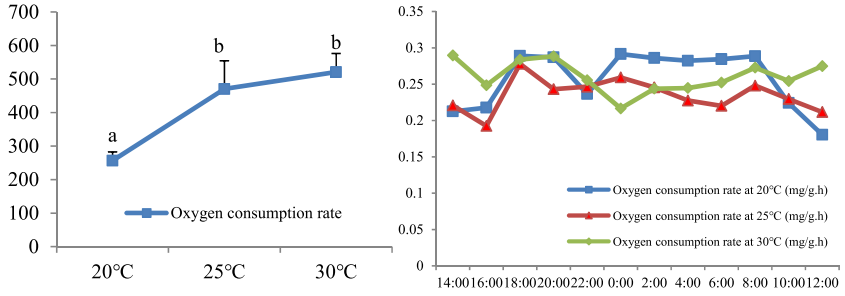

Fig. 3. Determination of the oxygen consumption rate and diurnal variation of the oxygen consumption rate of *C. alburnus* at 20 °C, 25 °C, and 30 °C. The error bars represent SEM and the columns marked with different letters indicate statistically significant differences ($P < 0.05$).

survival rate of *C. alburnus* decreased with increasing ammonia-nitrogen concentration and stress duration. The mortality at 24, 48, and 96 h in the high-concentration AN group was significantly higher than that in the other two groups ($P < 0.05$), and the mortality at 96 h in each group was significantly higher than that in the 24 and 48 h stress groups ($P < 0.05$) (Table 2).

3.2. Effects of DO and an on C. alburnus at the biochemistry level

3.2.1. Effect of hypoxia stress on antioxidant enzyme activity

The enzyme activity test results showed that the change curve of SOD activity with time increased gradually at first, reached a maximum at 12 h, was significantly higher than that at 0 h ($P < 0.05$), and then decreased, but was still higher than that at 0 h (Fig. 4A). The CAT activity remained stable at 6 h, increased, reached a maximum at 12 h, and then decreased (Fig. 4B). The curve of GSH activity level with time first increased gradually, reached a maximum at 12 h, then decreased, and roughly returned to the value at 0 h by 24 h (Fig. 4C). The change curve of MDA activity over time increased gradually without any significant decrease, and the value at 24 h was still significantly greater than that at 0 h ($P < 0.05$) (Fig. 4D). In conclusion, the change curves of antioxidant enzyme activities in the liver tissue under hypoxic stress reached a maximum at 12 h and then decreased.

3.2.2. Effect of an on antioxidant enzyme activity

The enzyme activity test results showed that changes in all enzyme activities in the control group were stable at a certain value. The change curve of SOD activity showed that the low and high AN groups showed a change trend of first increasing, then decreasing, and finally increasing. After reaching a maximum value at 6 h, it decreased rapidly to a minimum value at 12 h, and then increased slowly. The value at 96 h was close to that of the control group (Fig. 5A). The change curve of CAT activity showed that the low- and high-concentration AN groups also showed a change trend of first increasing, then decreasing, and then increasing. After reaching the maximum value at 12 h, it decreased rapidly to the minimum value at 48 h, and then increased slowly. The value at 96 h was still lower than that of the control group (Fig. 5B). The change curve of GSH activity showed that the low-concentration AN group showed a change trend of first increasing, then decreasing, and then increasing again. It decreased rapidly after reaching the maximum value at 12 h, decreased to the minimum value at 24 h, and then increased slowly. The value at 96 h was close to that of the control group. The high-concentration AN group showed a trend of first increasing and then slowly decreasing, reaching a maximum value at 12 h and then decreasing again, and the value at 96 h was lower than that in the control group (Fig. 5C). The change curve of MDA activity showed that the low- and high-concentration AN groups showed a change trend of first increasing, then decreasing, and then increasing, reaching the maximum value at 24 h, then decreased to the minimum value at 48 h, but was still higher than that of the control group, and finally increased (Fig. 5D). In summary, the change curve of all antioxidant enzyme activity under AN stress was similar; both began to show an upward trend and then showed a downward trend. In addition to MDA, the enzyme activity in the high-concentration group increased slightly and decreased significantly compared to that in the low-concentration group.

3.3. Effects of DO and an on C. alburnus at the molecular level

3.3.1. Transcriptome analysis under hypoxia

Sequencing samples of hypoxia 0 h_1, hypoxia 0 h_2, hypoxia 0 h_3, hypoxia 24 h_1, hypoxia 24 h_2, and hypoxia 24 h_3 yielded clean read counts of 24,089,479; 22,438,710; 21,075,012; 22,735,617; 22,869,021; and 24,067,212, respectively. The GC content of the six

Table 1
Mortality of fish under high AN stress for 96 h.

AN (mg/L)	Mortality for 96 h (%)
0	0.00 ± 0.00
10	6.67 ± 5.77
20	46.67 ± 5.77
30	60.00 ± 0.00

Table 2
The survival rate of fish under ammonia-nitrogen stress for 24–96 h.

AN (mg/L)	Mortality for 24 h (%)	Mortality for 48 h (%)	Mortality for 96 h (%)
0	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}
8	0 ± 0.00^{a}	0 ± 0.00^{a}	3.33 ± 5.77^{b}
16	3.33 ± 5.77^{b}	20.00 ± 10.00^{c}	36.67 ± 5.77^{d}

All data are presented as the mean ± SD. Different superscript letters indicate significant differences ($P < 0.05$).

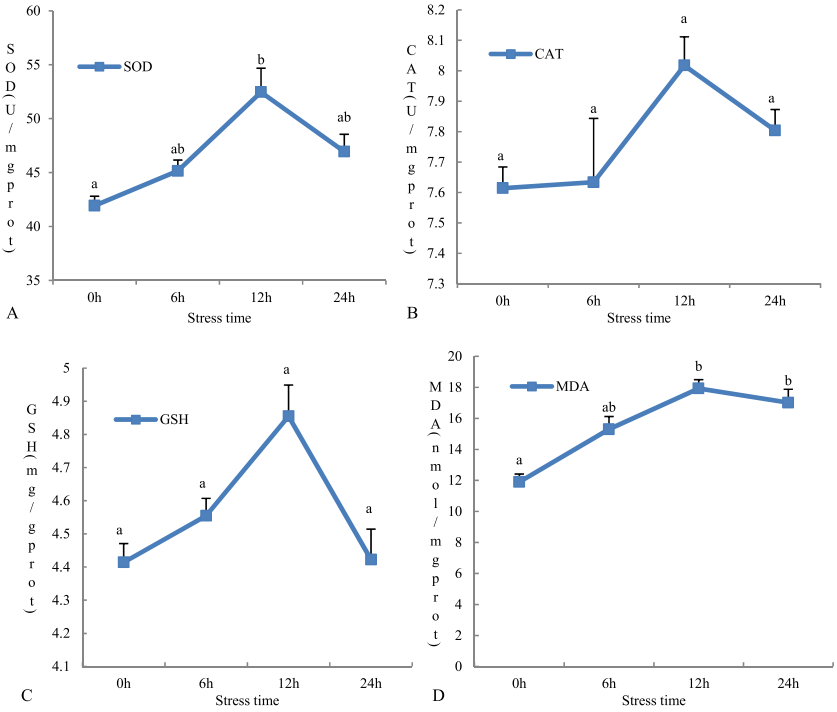

Fig. 4. The activities of SOD, CAT, GSH, and MDA for 0–24 h in *C. alburnus* (A. the activity of SOD, B. the activity of CAT, C. the activity of GSH, D. the activity of MDA). The error bars represent SEM and the columns marked with different letters indicate statistically significant differences ($P < 0.05$).

samples ranged from 46.65% to 47.25%. The proportion of total bases with a Phred value > 30 (the corresponding correct base recognition rate was 0.999) was 93.50–94.39%. Sequence alignment was performed between the clean data from each sample and the assembled transcript or the UniGene library. The reads were compared to the script or UniGene, and the mapped reads were used for subsequent analysis.

The number of DEGs were counted, screened, and clustered. The results showed significant differences in the expression of 428 genes before and after 24 h of hypoxia treatment, with 43 genes (10.05%) being upregulated and 385 genes (89.95%) being down-regulated. Based on the expression number of genes in different samples, the UniGene sequence was compared with the cluster of orthologous groups of proteins (COG), Gene Ontology (GO), Kyoto Encyclopaedia of Genes and Genomes (KEGG), clusters of orthologous groups for eukaryotic complete genomes (KOG), Pfam protein families (Pfam), Swiss-prot, evolutionary genealogy of genes (eggnog), and non-redundant protein (nr) databases using BLAST software to obtain the annotation information of UniGene. In total, 378 DEGs were annotated, including angiotensin-converting enzyme (*ACE*), zonadhesin-like nicotinamide riboside kinase, carboxypeptidase (*CP*), alpha-1,3/1,6-mannosyltransferase, and glycine-rich RNA-binding protein genes.

In total, 168 DEGs were successfully annotated using the GO database. These were classified into 59 secondary categories based on their biological processes, cellular components, and molecular functions. There were 24 secondary items based on biological processes, mainly focusing on cellular processes, single-organisation processes, metabolic processes, and biological regulation. Based on cell components, there were 18 secondary classification items, mainly focusing on cells, cell parts, organelles, membranes, membrane parts, and macromolecular complexes. Based on the molecular functions, there were 17 secondary classification items, mainly focusing on binding, catalytic, transporter, and nuclear acid-binding transcription factor activities.

In total, 183 DEGs were successfully annotated using the KEGG database. According to the KEGG metabolic pathway, the DEGs were divided into six branches: cellular processes, environmental information processing, genetic information processing, human disease, metabolism, and organismal systems. The cell process branch contained 26 genes (14.21%), of which the two pathways with

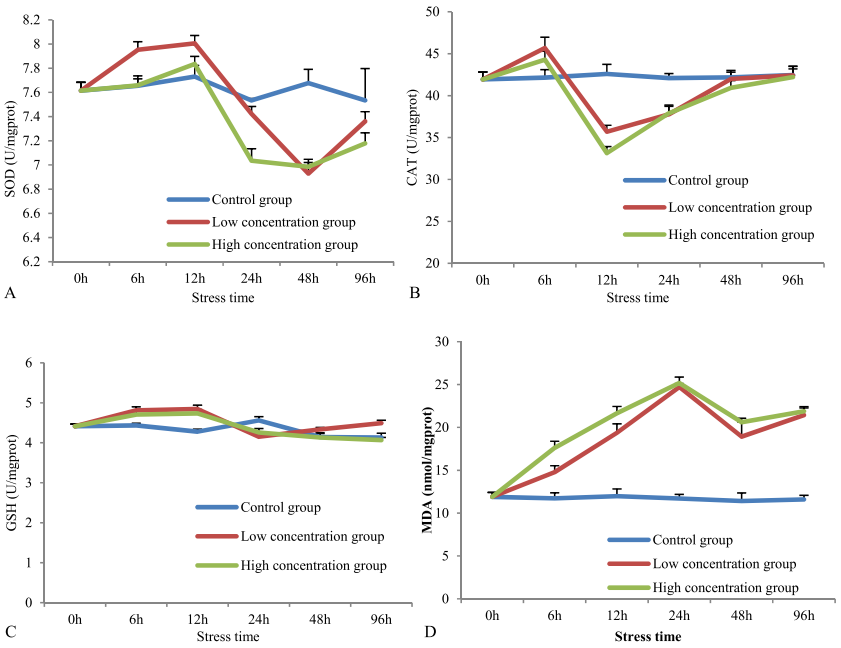

Fig. 5. The activities of SOD, CAT, GSH, and MDA for 0–96 h in *C. alburnus* (A. the activities of SOD, B. the activities of CAT, C. the activities of GSH, D. the activities of MDA). The error bars represent SEM and the columns marked with different letters indicate statistically significant differences ($P < 0.05$).

the largest number of genes were focal adhesion and regulation of the actin cytoskeleton, accounting for 30.77% and 19.23% of the total genes of the branch, respectively. The environmental information processing branch contained 24 genes (13.11%), of which the two pathways with the largest number of genes were ABC transporters and the MAPK signalling pathway, accounting for 29.17% and 20.83% of the total genes of the branch, respectively. The genetic information processing branch contained seven genes (3.83%), of which RNA degradation was the pathway with the largest number of genes, accounting for 42.86% of all genes in the branch. The human disease branch contained nine genes (4.92%), of which the most annotated pathway was *Salmonella* infection, accounting for 33.33% of all genes in this branch. The metabolic branch contained 79 genes (43.17%), of which the three pathways with the largest

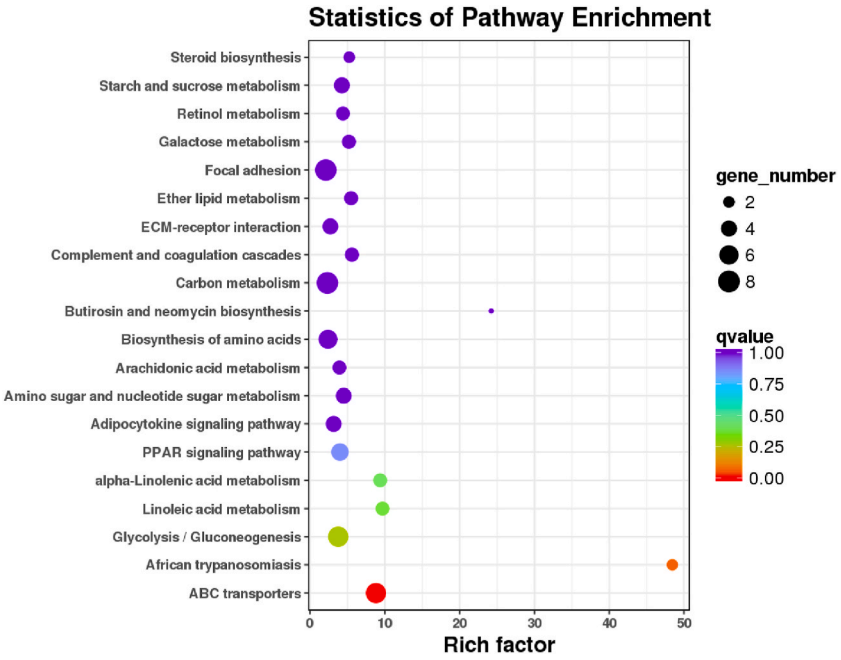

Fig. 6. Scatter diagram of the enrichment of DEGs in the KEGG pathway.

number of genes were carbon metabolism, glycolysis/gluconeogenesis, and amino acid biosynthesis, accounting for 10.13%, 8.86%, and 7.59% of the total genes of the branch, respectively. The organismal system branch contained 17 genes (9.29%), of which the two pathways with the largest number of genes were the peroxisome pro1iferator-activated receptor signalling pathway and the insulin signalling pathway, accounting for 29.41% of the total genes of the branch.

Different genes perform different biological functions in coordination with each other. *ACE* and *CP* were major DEGs. The primary biochemical metabolic and signal transduction pathways used by the DEGs before and after hypoxia stress included ABC transporters, linoleic acid metabolism, and α-linoleic acid metabolism. The DEGs were mainly enriched in the ABC transporter signal transduction pathway (Fig. 6).

3.3.2. *Transcriptome analysis under an stress*

AN stress 0 h1, 0 h2, 0 h3, 96 h1, 96 h2, and 96 h3 yielded clean read counts of 23,099,903; 21,527,949; 20,427,906; 20,597,831; 22,198,061; and 20,520,490, respectively. The GC content of the six samples ranged from 46.64% to 47.41%. The proportion of total bases with a Phred value > 30 (the corresponding correct base recognition rate was 0.999) was 93.01–94.41%. Sequence alignment was performed between the clean data from each sample and the assembled transcript or UniGene library. The reads were compared to the script or UniGene and the mapped reads were used for subsequent analysis. The number of DEGs were counted, screened, and clustered.

The results showed that there were significant differences in the expression of 65 genes before and after 96 h of treatment, with 36 genes (55.38%) being upregulated and 29 genes (44.62%) being downregulated. Based on the expression number of genes in different samples, the UniGene sequence was compared with COG, GO, KEGG, KOG, Pfam, Swiss-prot, eggnog, and nr databases using BLAST software to obtain the annotation information of UniGene. A total of 34 DEGs were successfully annotated in the GO database, and 22 DEGs were successfully annotated in the KEGG database.

Serum/glucocorticoid-regulated kinase (*SGK*), stearoyl-CoA desaturase (*SCD*), or 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*) genes were the major DEGs. Different genes perform different biological functions in coordination with each other. The primary biochemical metabolic and signal transduction pathways used by the DEGs before and after ammonia-nitrogen stress included steroid biosynthesis and glycine, serine, and threonine metabolism transporter signal transduction pathways (Fig. 7). The DEGs were mainly enriched in steroid biosynthesis and glycine, serine, and threonine metabolism transporter signal transduction pathways.

3.3.3. *qRT-PCR validation*

The *ACE* and *CP* genes were major DEGs under hypoxic stress, and *SGK*, *SCD*, and *HMGCR* genes were major DEGs under high AN stress. Next, the results were validated by qRT-PCR. They showed that the detected gene expression trends were consistent with the transcriptome expression analysis results, proving that the RNA-seq results were reliable (Fig. 8).

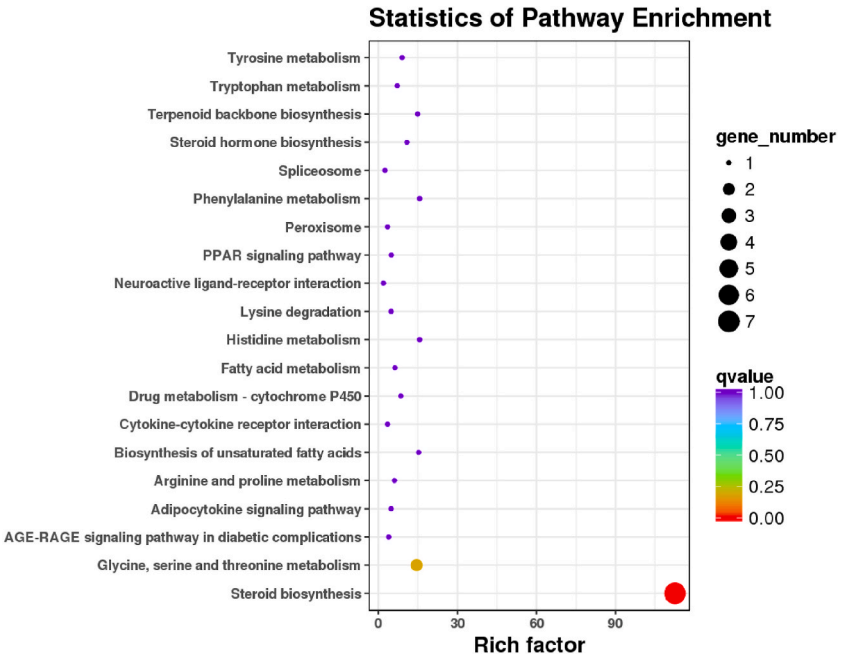

Fig. 7. Scatter diagram of the enrichment of DEGs in the KEGG pathway.

4. Discussion

In this study, the results indicating that with an increase in temperature, the tolerance of fish to hypoxia decreased. Therefore, special attention should be paid to floating heads caused by hypoxia during fish breeding and transportation. For example, to avoid fish floating heads, the DO level must be maintained above 2.195, 1.540, and 1.175 mg/L at a water temperature of 30 °C, 25 °C, and 20 °C, respectively. Moreover, the oxygen consumption rate of fish indicated a circadian rhythm [20]. Some studies have categorised the circadian variation in fish metabolic levels into three types: day > night, night > day, and an insignificant difference between day and night [21]. Interestingly, in this experiment, at 20 °C and 25 °C, the oxygen consumption rate of the fish was higher at night and lower during the day. However, the situation changed at 30 °C, and the oxygen consumption rate was higher during the day, with the lowest value occurring at 00:00 at night. Some studies have suggested that variations in the oxygen consumption rate represent the inherent activity cycle of fish in nature. For example, a high oxygen consumption rate represents a period of feeding or other activities [22].

C. alburnus in its natural state is a large and ferocious carnivorous freshwater fish that mainly feeds on live fish, with the ferocious character of actively chasing other fish for food and prey day and night. The results of this study are consistent with *C. alburnus* catching prey during both the day and night. However, at different temperatures, the fish showed different living habits because the oxygen consumption rate was higher at night at 20 °C and 25 °C, indicating that fish hid in the day and came out at night. However, at 30 °C, the lower oxygen consumption rate at night indicated that fish hid at night and appeared during the day. Therefore, during the breeding process of *C. alburnus*, water should be changed and the aerator should be opened frequently to maintain sufficient and balanced DO levels during the day and night to meet the oxygen demand of fish for continuous swimming, predation, and other physiological activities.

Studies have shown that, as the concentration of AN increases, the mortality rate of *Esox lucius* (barracuda) also increases. Moreover, with the prolongation of poisoning time at various AN concentrations, the mortality rate of fish showed an increasing trend. The toxic effect was positively correlated with the concentration and poisoning time of AN [23]. Similar conclusions were drawn from the present study, where the survival rate of the high-AN group was significantly lower than that of the other two groups. The SC of *C. alburnus* was 2.41 mg L^{-1}, which surpasses the requirement of total AN based on China's fishery water quality standard, which states that it should not exceed 2 mg L^{-1} [23]. However, further studies are needed to determine whether long-term cultivation at this concentration has adverse effects on the physiological function of the fish. Some studies have also proposed that the baseline continuous concentration range for total AN in freshwater environments suitable for aquatic organisms is 0.0664–3.92 mg/L [24]. Therefore, to maintain the healthy growth of fish and normal respiratory and detoxification metabolic functions, close monitoring of the AN concentration in aquaculture water bodies, regulating water quality in a timely manner, and reducing aquaculture risks are recommended.

It is generally believed that SOD plays an important role in scavenging reactive oxygen species before other antioxidant enzymes, such as CAT [2]. In the present study, it was observed that under hypoxic stress, liver SOD activity first gradually increased and reached a maximum at 12 h, which is similar to that reported by Lushchak et al. [25], wherein fish increased the activity of some antioxidant enzymes in advance under hypoxic stress in the absence of oxygen or hypoxia. CAT activity first showed a stable period and then increased, indicating that the reaction of SOD occurred earlier than that of CAT, which is consistent with the experimental results of Ou et al. [2]. However, some studies have shown that CAT activity in fish liver changes differently after hypoxic stress. Lushchak et al. found that CAT activity in *Perccottus glenii* (ruffe) decreased significantly after hypoxic stress, suggesting that the large number of free radicals produced by lipid oxidation inhibited CAT activity [26]. Lushchak et al. found that the CAT activity in *Cyprinus carpio* (common carp) and *Carassius auratus* (goldfish) remained stable after hypoxic stress [27,28]. Wang et al. found that the CAT activity in *Sepiella maindroni* (cuttlefish) first increased and then decreased after hypoxic stress [29]. This indicates that CAT has strong species specificity, and its role in biological hypoxia tolerance requires further study.

GSH helps fish cope with oxidative stress by removing free oxygen radicals. Liu et al. reported that GSH synthesis begins during oxidative stress. The level of GSH in this study recovered after increasing under hypoxic stress, indicating that hypoxic stress caused oxidative pressure in the fish and the increase in GSH helped remove excess free radicals produced by the body [30]. This observation was consistent with the experimental results reported by Ou et al. [2]. The level of MDA, the final product of lipid peroxidation, accurately reflects the degree of lipid peroxidation in the body [31]. In this study, MDA activity did not decrease significantly after increasing acute hypoxic stress, indicating that the effect of hypoxic stress on free radicals in the liver did not recover.

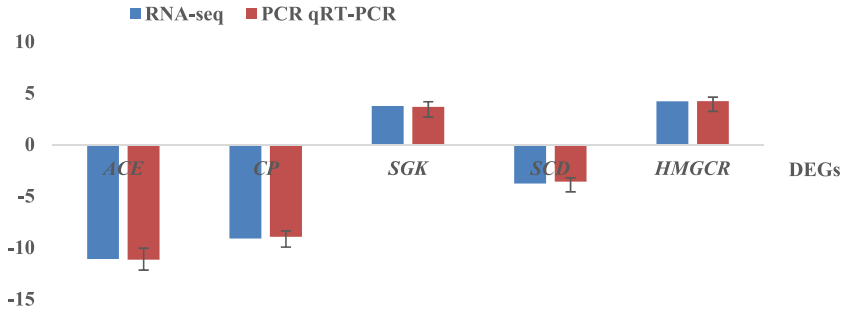

Fig. 8. Comparison of seven DEGs by qRT-PCR and transcriptome analysis.

In conclusion, these results showed that the body first removed excess free radicals by improving antioxidant enzyme activity to deal with the pressure of oxidative stress in a hypoxic environment before 12 h. However, with the extension of stress time, which affects the ability of the body to remove excess free radicals, antioxidant enzyme activity decreased significantly. Therefore, during the production process, it is recommended that fish not be kept in low-oxygen environments for more than 12 h.

However, similar to the results of the hypoxia stress experiment, the change curve of antioxidant enzyme activity under AN stress was similar; both began to show an upward trend followed by a downward trend. At the same time, in addition to MDA, the enzyme activity in the high-concentration group increased slightly and decreased significantly compared to that in the low-concentration group, and the enzyme activity decreased after 96 h. In the present study, the activity of the antioxidant enzyme SOD increased in the later stages of AN stress, indicating that AN stress did not produce irreversible toxic effects on the liver. It was speculated that the low-concentration of AN were more beneficial for inhibiting the production of oxidative free radicals than the high-concentration group, as they impacted enzyme activity, suggesting that in daily aquaculture production, close attention should be paid to the concentration of AN in the water body to prevent excessive AN from causing imbalances in the antioxidant system of fish, affecting their survival rate.

In this study, the *ACE* and *CP* genes were the primary up- and down-regulated DEGs. *ACE* is an important part of the renin-angiotensin system, a zinc-containing metalloproteinase that binds to the cell membrane and plays an important role in regulating blood pressure, electrolyte and body fluid balance, cardiovascular system development, and structural remodelling. Its structure is similar to that of protein carboxylase A [32]. Moreover, these polymorphisms are closely associated with the occurrence and development of various cardiovascular and cerebrovascular diseases [33]. *CP* catalyses the hydrolysis of carboxyl-terminal amino acids in polypeptide chains [34] and plays essential roles in different activities, ranging from food digestion to the synthesis of neuroendocrine peptides. Any change in its enzymatic activity affects the nutritional metabolism of larvae. For instance, a decrease in its secretion or enzyme activity leads to a decline in digestive ability, affecting the growth of the larvae [34,35].

The enrichment scatter plot of DEGs in the KEGG pathway showed that ABC transporters are one of the primary biochemical, metabolic, and signal transduction pathways associated with the DEGs before and after hypoxic stress. The ABC transporter gene family proteins used the energy generated by ATP cleavage to transport a variety of substrates to the membrane and participate in a variety of biological processes, such as nutrient intake, cell detoxification, lipid homeostasis, signal transduction, viral defence, and antigen presentation [36–39].

Considering the changes in the expression of these two genes and the biochemical, metabolic, and signal transduction pathways, it is suggested that it is necessary to focus on the blood pressure, electrolyte and body fluid balance, cardiovascular system, nutritional metabolism, and digestive ability of fish in a hypoxic environment.

The *SGK*, *SCD*, and *HMGCR* genes were the major DEGs. *SGK* is a member of the serine/threonine protein kinase family. Its transcription, activity, and subcellular localisation are regulated by various intracellular and extracellular stimuli. It is a functional intersection of various intracellular signalling pathways and is involved in cell proliferation, osmotic regulation, ion channel regulation, cell survival, and apoptotic responses, which are closely related to diseases [40,41]. In addition, *SGK* is involved in regulating K^+ and Cl^- ion channels, Na^+/K^+-ATP activity, and Na^+-H^+ exchange. Therefore, *SGK* may play an important role in physiological activities, such as the regulation of cell volume and osmotic pressure, and the maintenance of ion balance [42]. *SCD* is a membrane protein of the endoplasmic reticulum that catalyses the conversion of saturated fatty acids into monounsaturated fatty acids. It is a key regulator of energy metabolism, plays an important role in dyslipidaemia, and participates in the regulation of cell growth and differentiation [43]. *HMGCR* is a key enzyme in endogenous cholesterol synthesis in the liver and can be used as an immunological marker of immune necrotising myopathy [44,45]. Chen et al. suggested that HMGCR degradation and SREBP-2 cleavage are the two major feedback regulatory mechanisms governing cholesterol biosynthesis. Lanosterol is a bona fide endogenous regulator that specifically promotes HMGCR degradation, and other C4-dimethylated sterol intermediates may also regulate HMGCR degradation and SREBP-2 cleavage [45].

Therefore, *SGK*, *SCD*, and *HMGCR*, as the main DEGs, are closely related to disease; osmotic ion balance; the apoptotic response; dyslipidaemia; energy metabolism; steroid biosynthesis; and the glycine, serine, and threonine metabolic signal transduction pathways. The DEGs were mainly enriched in steroid biosynthesis and glycine, serine, and threonine metabolic signal transduction pathways. This shows that it is necessary to focus on disease prevention in *C. alburnus* in high-AN environments by looking out for an abnormal osmotic ion balance as well as blood lipid and energy metabolism disorders. In conclusion, high AN levels have a significant effect on *C. alburnus*, and particular attention should be paid to fish under these conditions.

5. Conclusion

In this study, the effects of DO and AN levels on *C. alburnus* were analysed at three levels: physiology, biochemistry, and molecular. The following conclusions were drawn: 1) physiological level: The DO level must be maintained above 2.195, 1.540, and 1.175 mg/L at water temperatures of 30 °C, 25 °C, and 20 °C, respectively. At 20 °C or 25 °C, the *C. alburnus* fish hides during the day and appears at night, whereas at 30 °C, it hides at night and appears during the day. The survival rate of the high-AN group was significantly lower than that of the other two groups. 2) Biochemistry level: The change curves for antioxidant enzyme activity showed that fish should not be maintained in a low-oxygen environment for more than 12 h. It was recommended that fish not be kept in low-oxygen environments for more than 12 h. The increase and decrease in enzyme activity (except that of MDA) in the high-AN group were less than those in the low-AN group, and the enzyme activity was lower after 96 h. 4.It was speculated that the low-concentration of AN were more beneficial for inhibiting the production of oxidative free radicals than the high-concentration group. 3) Molecular level: Considering the changes in the expression of *ACE* and *CP* as well as the biochemical, metabolic, and signal transduction pathways, it is necessary to focus on the

blood pressure, electrolyte and body fluid balance, cardiovascular system, nutritional metabolism, and digestive ability of fish in hypoxic environments. *SGK*, *SCD*, and *HMGCR* genes were the major DEGs under high-AN stress, and were mainly enriched in steroid biosynthesis or glycine, serine, and threonine metabolism transporter signal transduction pathways. In short, low DO or high AN levels significantly affect the survival, activity, liver antioxidant capacity, and expression of some genes of *C. alburnus.* Therefore, it is necessary to focus on the DO and AN during *C. alburnus* breeding.

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Compliance with ethical standards

This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

Code availability

Software application.

Data availability

The authors are unable or have chosen not to specify which data has been used.

CRediT authorship contribution statement

Cheng Shun: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jiang Wen-ping:** Project administration, Methodology, Investigation. **Liu Shi-li:** Validation, Supervision, Software, Methodology. **Zheng Jian-bo:** Supervision, Software, Resources, Methodology. **Chi Mei-li:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Hang Xiao-ying:** Visualization, Validation, Software, Methodology, Conceptualization. **Peng Miao:** Visualization, Investigation, Formal analysis. **Li Fei:** Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:The authors reports was provided by Zhejiang Institute of Freshwater Fisheries. The authors reports a relationship with Zhejiang Institute of Freshwater Fisheries that includes: board membership. Li Fei has patent pending to Licensee. The authors declare that there are no competing interests. If there are other authors, they 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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