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Effects of Ammonia and Nitrite Stress on Immune and Metabolic Responses of Japanese Sea Bass, *Lateolabrax Japonicus*

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Abstract

We investigated the effects of ammonia and nitrite stress on genes involved in metabolism and disease resistance in Japanese sea bass (*Lateolabrax japonicus*). Fish were exposed to pure seawater (control), 65.4 mg/L NH_4^+-N, or 60.9 mg/L NO_2^--N for 7 days. Muscle, head-kidney, gill, spleen, and liver tissues were sampled regularly and the expression of immune and metabolism-related genes was monitored. The results indicated that the expression of immune-related genes such as heat shock proteins 70 and 90 were upregulated in the head-kidney, spleen, gill, liver, and muscle tissues under ammonia and nitrite stress. Glutamine synthetase (*GS*) expression increased to different degrees under ammonia and nitrite stress, while glutamate dehydrogenase (*GDH*) in the stress group upregulated in the gills. The expression of carbonic anhydrase (*CA*) was upregulated in the spleen and gill tissue under ammonia or nitrite stress. Expression of leptin (*LEP*) was upregulated in the muscle, gills and liver under nitrite stress; under ammonia stress, LEP expression did not fluctuate significantly in the spleen or gill. Insulin-like growth factor-1 (*IGF-1*) was downregulated in the spleen, while did not fluctuate obviously in the muscle and head-kidney under ammonia stress. These results indicate that ammonia and nitrite stress led to an upregulated immune related indexes response and increased metabolic activities in *L. japonicus*, and provides reference suggestions for response status of these health markers answering the outside environment intimidation.

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Introduction

Japanese sea bass (*Lateolabrax japonicus*) are an important aquaculture species in China, with an annual production exceeding 100,000 tons statistically according to the 2019 China fisheries statistics yearbook. High-intensity aquaculture is often used to meet demands. As a result, a negative effect on the farm health status and production is caused by high concentrations of ammonia and nitrite (Randall et al., 2002; Hargreaves et al., 2001; Wei et al., 1993). High ammonia concentration can increase the pH and reduce the oxygen carrying capacity of the blood, inhibit the respiratory function of the gill and damage the liver and kidney system (Randall et al., 2002). Thus, ammonia can cause oxidative damage and ion concentration imbalances, resulting in slow growth, poor physical fitness, reduced stress adaptation, and mortalities (Benli et al., 2008; Sinha et al., 2014). Nitrite mainly oxidizes ferrous hemoglobin to methemoglobin in the blood, reduces the number of red blood cells and hemoglobin, and weakens oxygen transport. NO_2^--N can be absorbed across the gill epithelium and accumulate gradually in blood, resulting in asphyxia, methemoglobinemia (Moraes et al., 2015; Tucker et al., 1989). Therefore, understanding of the immune and metabolic response mechanisms will benefit the development of health management of sea bass aquaculture.

Altering regulation of various fish genes respond to environmental changes. Genes such as leptin (LEP) and insulin-like growth factors-1 (IGF-1) related to growth participate in appetite and energy regulation (Pelleymounter et al., 1995) as well as affect embryonic development and differentiation (Duan, 2003) induced by environmental stress. Genes of the enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) in organic nitrogen metabolism and detoxification play central roles in maintaining normal tissue nitrogen levels. Carbonic anhydrase (CA) is involved in carbon metabolism as well as ion exchange and transport. Heat shock proteins 70 (Hsp70) and 90 (Hsp90) canprotect proteins from denaturation, assist in repairing the damaged proteins, while serving as indicators of physiological status (Vijayan et al., 2006), immunity (Young, 1992), and biomarkers of water quality change (Halloran et al., 2000; Pomerai., 1996). Very little is currently known about the effects of ammonia and nitrite stress on growth metabolism and immune function of *L. japonicus*.

The purpose of this study was to explore the expression of seven functional genes, including those related to immunity as well as metabolism capacity in *L. japonicus* under ammonia and nitrite stress. These results will provide effective biomarkers and crucial reference value to respond to NH_4^+-N and NO_2^--N stress in molecular level for *L. japonicus* healthy aquaculture.

Materials and Methods

Animals

Healthy *L. japonicus* of 33.71 ± 2.39 g and 12.63 ± 0.38 cm were collected from a local fish farm and reared in tanks at Shenzhen Base, South China Sea Fisheries Research Institute of the Chinese Academy of Fishery Sciences (Shenzhen, China). Fish were acclimatized in filtered aerated seawater at salinity of 10, pH 8.2, and 28 ± 2°C for 7 days. Water quality parameters including salinity, NH_4^+-N, NO_2^--N, and temperature were measured daily. One-third of the water in each tank was renewed daily, and water quality factors are consistent with the initial value. Filtration mesh was cleaned and remaining food and excrement were regularly removed. Fish were fed three times a day with commercial seabass buoyant feed until 24 hours prior to the experiment. The experiments were complied per the current animal-care laws in China.

Ammonia and nitrite stress test

After the acclimation period, a preliminary experiment was conducted to measure concentrations of NH_4^+-N and NO_2^--N causing median lethal concentration (LC_{50}) within 96 hours and to determine upper limit of NH_4^+-N and NO_2^--N concentrations; Pure seawater with no ammonia and nitrite as control group, modified according to previous method (Han et al., 2014). Nine 200 L tanks groups were prepared, including a control, one with 65.4 mg/L NH_4^+-N, and one with 60.9 mg/L NO_2^--N, with three replicates per group and fifteen fish per tank. Measure water quality daily, and add NH_4Cl and $NaNO_2$ to keep these concentrations. These fish were maintained for one week under acute stress ammonia and nitrite. The stability of the water was continuously maintained using a portable multiparameter meter (YSI, USA). Three fish from each tank were randomly sampled at day 1, 3, 5, and 7 and the head-kidney, spleen, liver, muscle, and gills were collected. Tissues were preserved in RNA Later and frozen at −70°C.

RNA extraction, cDNA synthesis, and RT-qPCR analyses

Total RNA was extracted from about 80 mg ~ 100 mg samples using 1ml Trizol (Invitrogen, USA) following the manufacturer's instructions. 60ng/μl RNA was used for RT-qPCR. Sample purity was verified by measuring absorbance at 260 nm and 280 nm using a NanoDrop 2000 (USA) and sample integrity was confirmed using 1.5% agarose gel electrophoresis. RNA was dissolved in RNase-free water and stored at −70°C until use. cDNA was synthesized using the TransScript cDNA Synthesis SuperMix (TransGen Biotech, AT301, Guangzhou, China) according to instructions. RT-qPCR was performed using the SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan) with a Roche LightCycler® 480 system (USA) to quantitate the expression of seven target genes. Specific primer sequences were designed based on the open reading frame (ORF) of the target genes using Primer Premier 5.0 software (**Table 1**). The β-actin gene of *L. japonicus* was used as an internal control to verify successful reverse transcription and calibrate the cDNA template. Specific primer efficiency was evaluated using an amplification plot and melting curve. RT-qPCR was carried out in a total volume of 10 μL containing 5 μL SYBR® Premix Ex Taq™ II (2 ×) (TaKaRa), 2 μL 1:6 diluted cDNA, 0.2 μL each of 10 μmol/L forward and reverse primers (**Table 1**), and 2.6 μL DEPC-treated water. Amplification took place at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 55-60°C (depending on the primers used) for 20 s, and extension for 30 s at 72°C. Melting curve analysis was implemented and results were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

The value of each variable was expressed as the mean ± standard error (SE). GraphPad Prism v.8.0.1 was used to carry out two-way ANOVA or mixed model analyses. All changes in gene expression were compared to baseline levels measured in the control group. Differences controls were regarded as significant when $P < 0.05$.

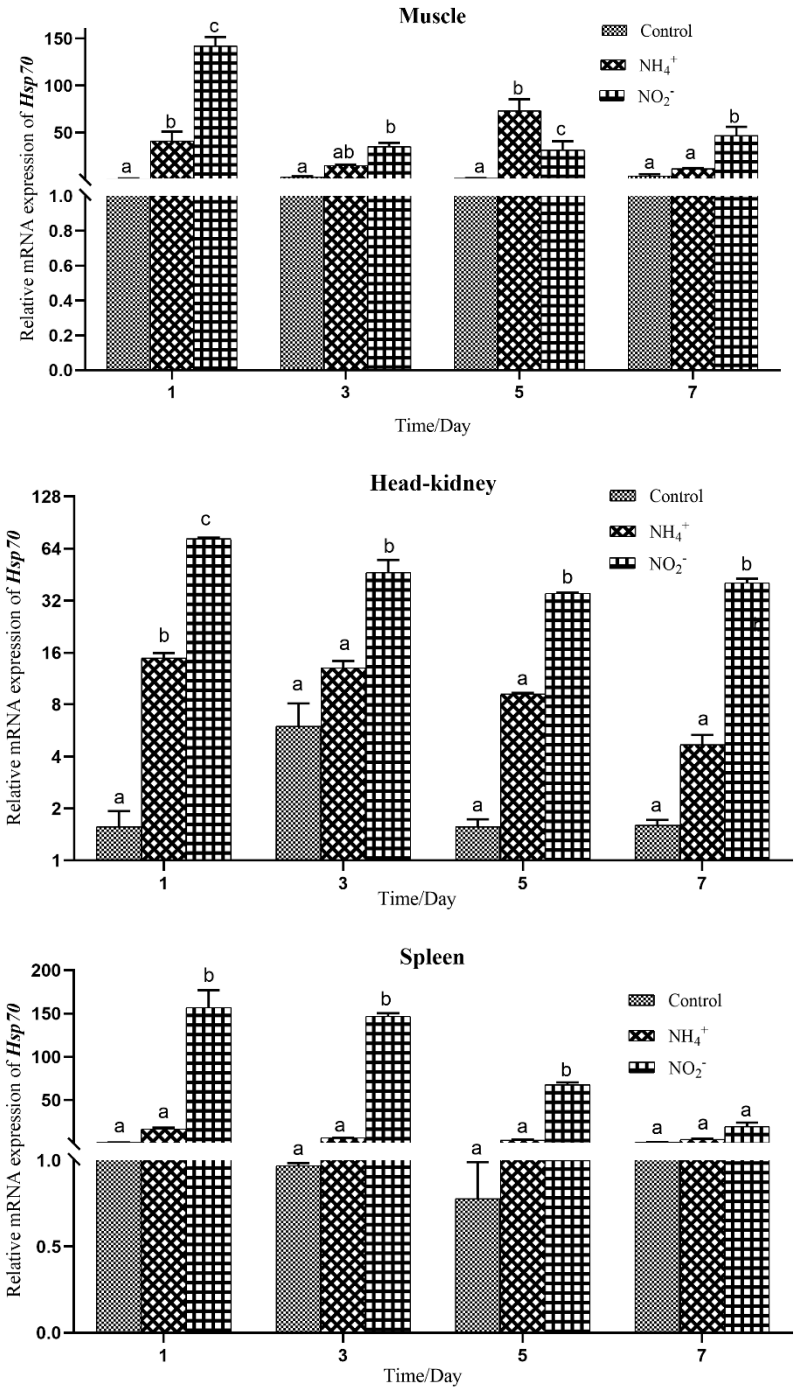
Table 1 The primers used for cloning and expression analysis

Name	Target	Sequence (5'→3')	GenBank accession number
Lep-RT-F	Real-time PCR	GAGCAGCTCATGGTCAGG	KF850511
Lep-RT-R	Real-time PCR	GGCCACTATGGAGGAAGGT	
IGF-1-RT-F	Real-time PCR	CGCAATGGAACAAAGTCGGAATAT	JN596878
IGF-1-RT-R	Real-time PCR	GTGAGAGGGTGTGGCTACAGGAGA	
HSP70-RT-F	Real-time PCR	ACGGCATTTTGAACGTGTCC	MN842806
HSP70-RT-R	Real-time PCR	GCAGCGATCTTCTCCCTCTG	
HSP90-RT-F	Real-time PCR	GGATGAGGACGACAAGAA	MN842808
HSP90-RT-R	Real-time PCR	GTTGGAGACTGTGACCTT	
CA-RT-F	Real-time PCR	CACCTGGAATTTCTCTGGATGA	MN842805
CA-RT-R	Real-time PCR	TTTGCACTGGCTGGATGTCTCT	
GS-RT-F	Real-time PCR	GACTTCGGTGTGGTGGTGTCCTT	MN842809
GS-RT-R	Real-time PCR	CATCCTCTCGCATCTCCTTTGTG	
GDH-RT-F	Real-time PCR	CTCTGACACTGAGCTTGAGAAAATC	MN842807
GDH-RT-R	Real-time PCR	GGTGGTGGCGAAGGTGTCTG	
β-actin-F	Real-time PCR	CAACTGGGATGACATGGAGAAG	HE577671
β-actin-R	Real-time PCR	TTGGCTTTGGGGTTCAGG	

Results

Immune function-related gene expression

Expression of Hsp70 and Hsp90 showed an upward trend in the head-kidney, spleen, gill, liver, and muscle tissues all the time points under acute stress from NO_2^-N; At the first days of NH_4^+-N stress they also showed upregulation. The expression of Hsp70 was higher under nitrite stress than under NH_3-N stress. With the exception of the muscle samples taken on day 5, Hsp90 was more highly expressed under NO_2^--N stress than NH_4^+-N stress (**Figures 1 and 2**).


Muscle
Control
NH_4^+
NO_2^-
Relative mRNA expression of *Hsp70*
Time/Day
Head-kidney
Control
NH_4^+
NO_2^-
Relative mRNA expression of *Hsp70*
Time/Day
Spleen
Control
NH_4^+
NO_2^-
Relative mRNA expression of *Hsp70*
Time/Day

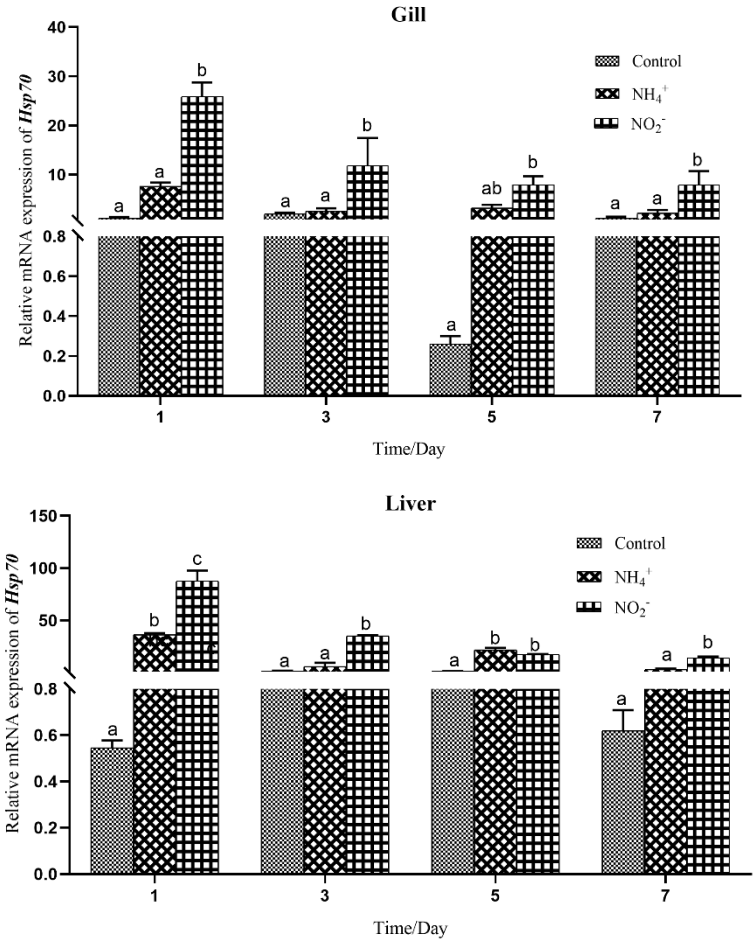
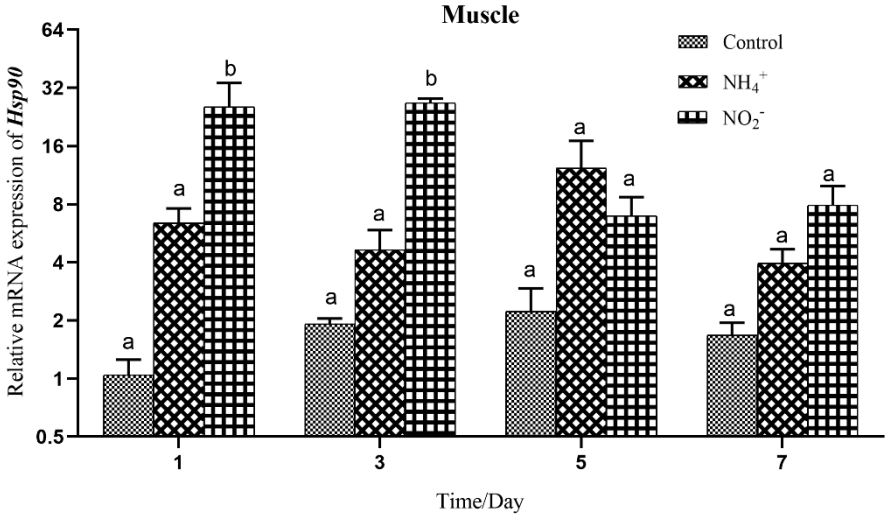
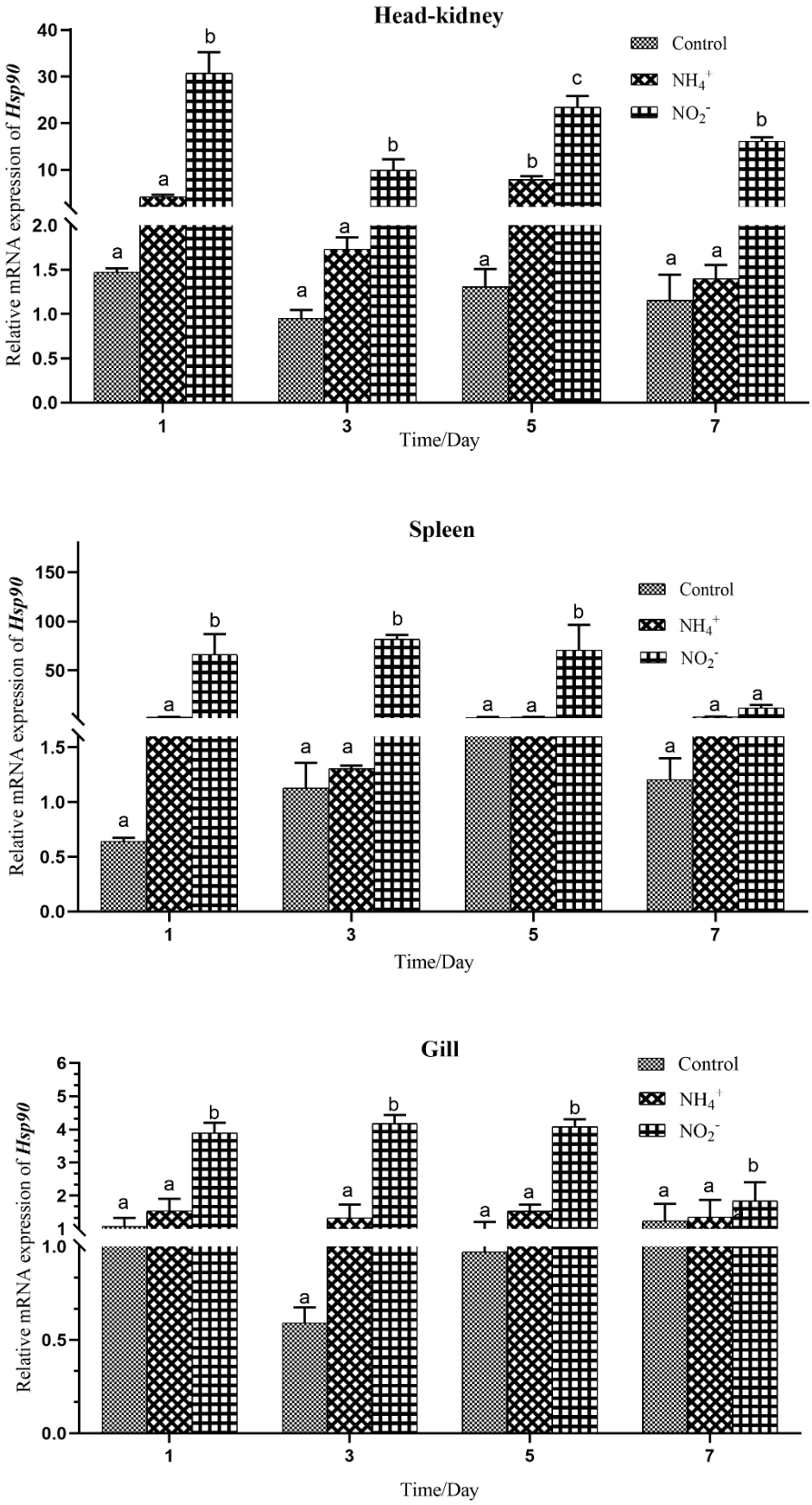


Figure 1 Hsp70 gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.




Head-kidney
Relative mRNA expression of Hsp90
Control
NH4+
NO2-
Time/Day
Spleen
Relative mRNA expression of Hsp90
Control
NH4+
NO2-
Time/Day
Gill
Relative mRNA expression of Hsp90
Control
NH4+
NO2-
Time/Day

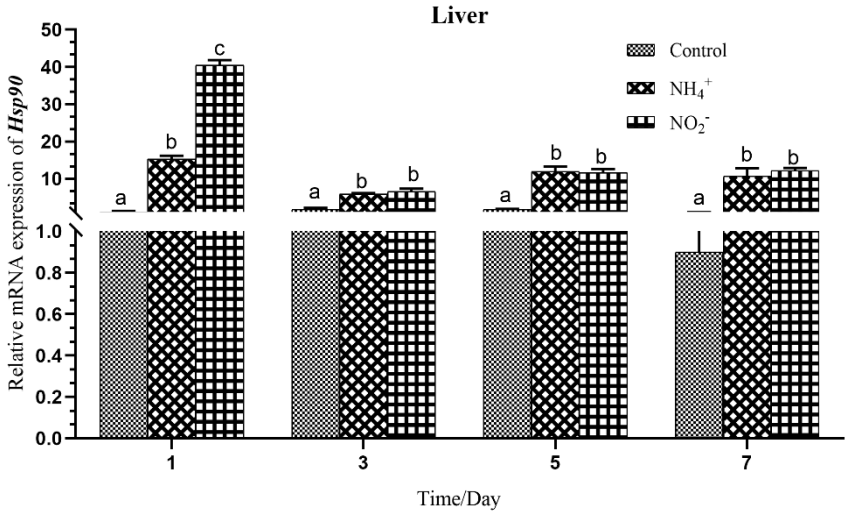
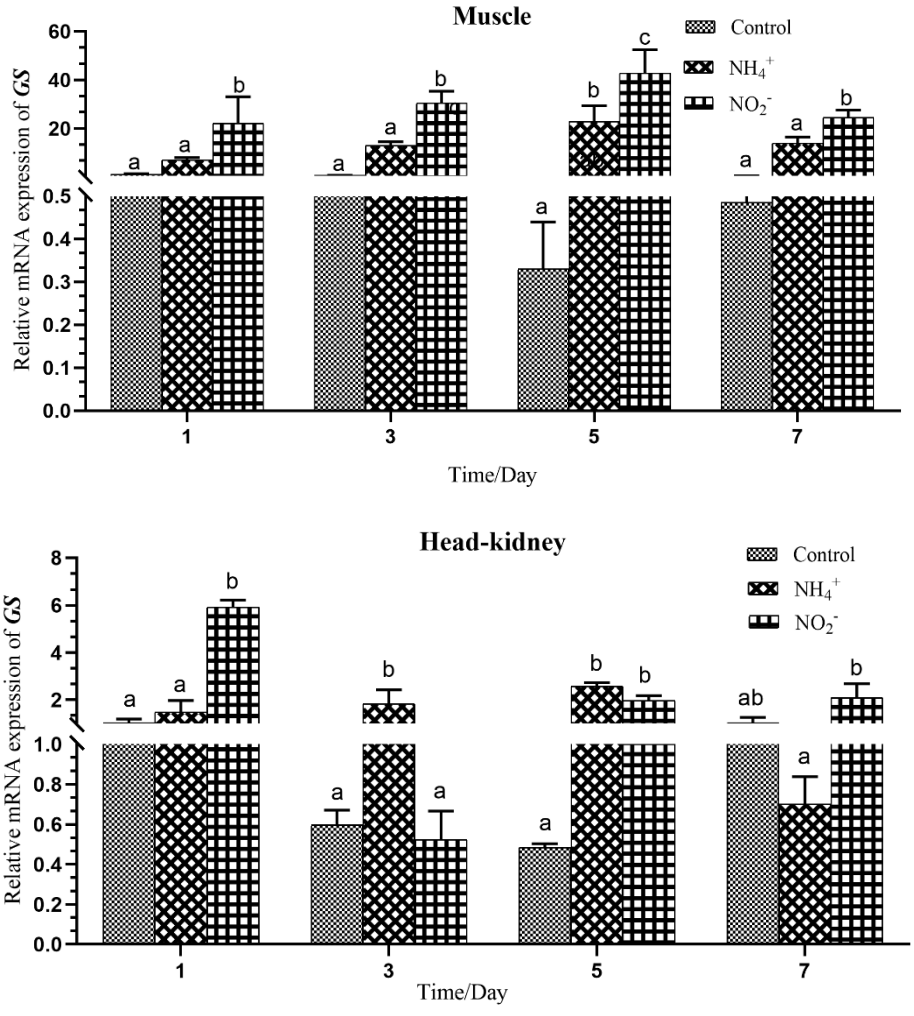


Figure 2 Hsp90 gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.

Organic nitrogen metabolism-related gene expression

GS was upregulated in the spleen, gill, liver, and muscle under NH_4^+-N and NO_2^--N stress. In the head-kidney, it was first upregulated and then reduced to control levels by day 7 under NH_3-N stress (**Figure 3**).



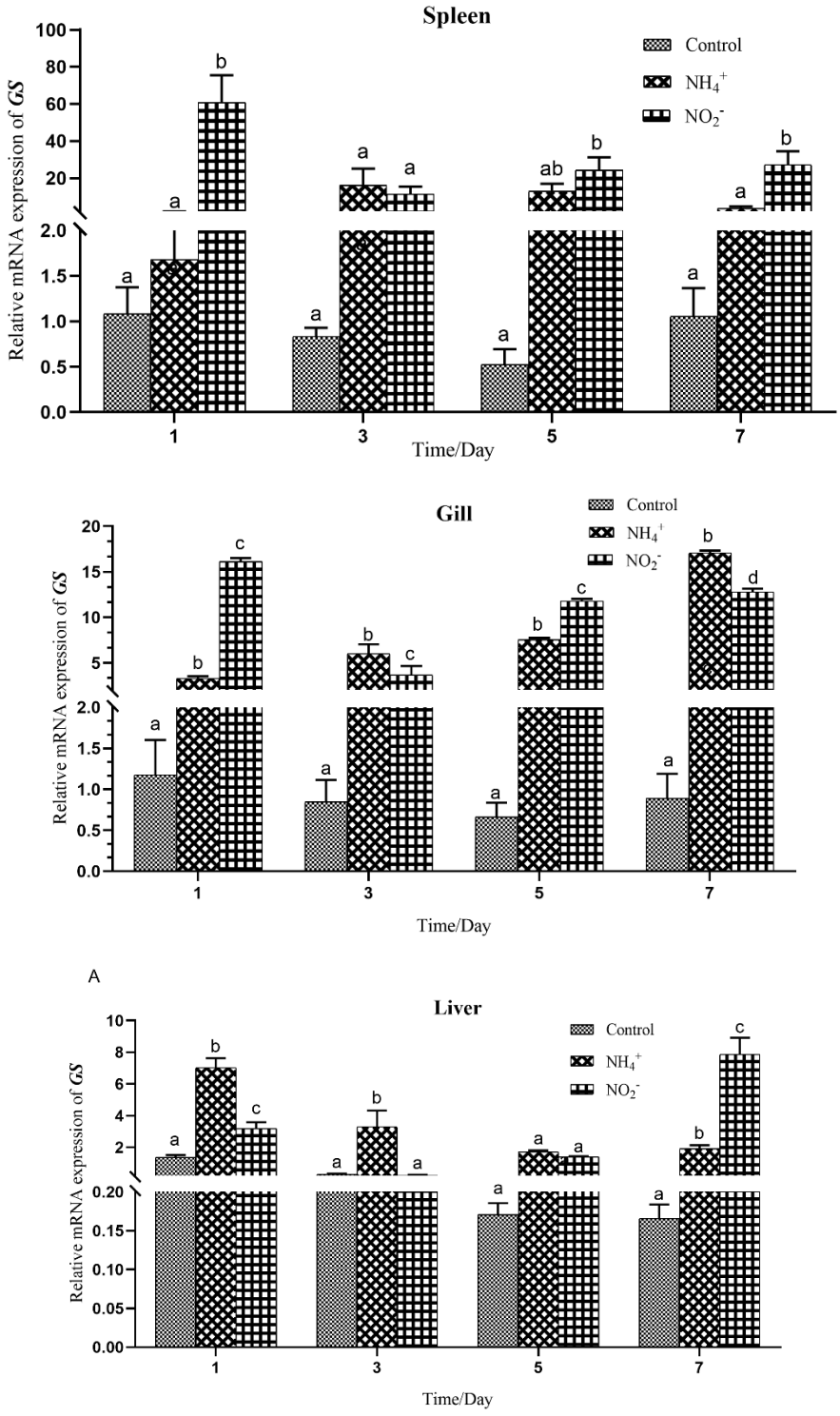
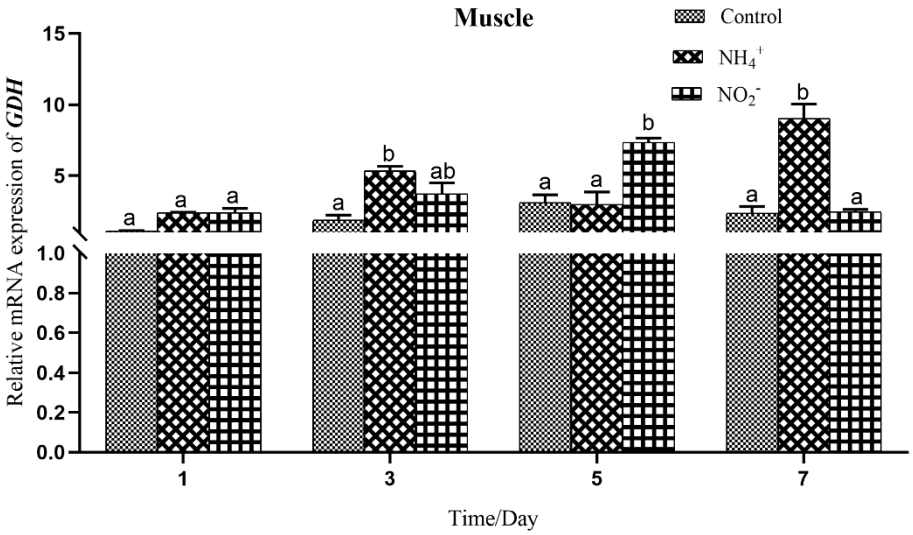
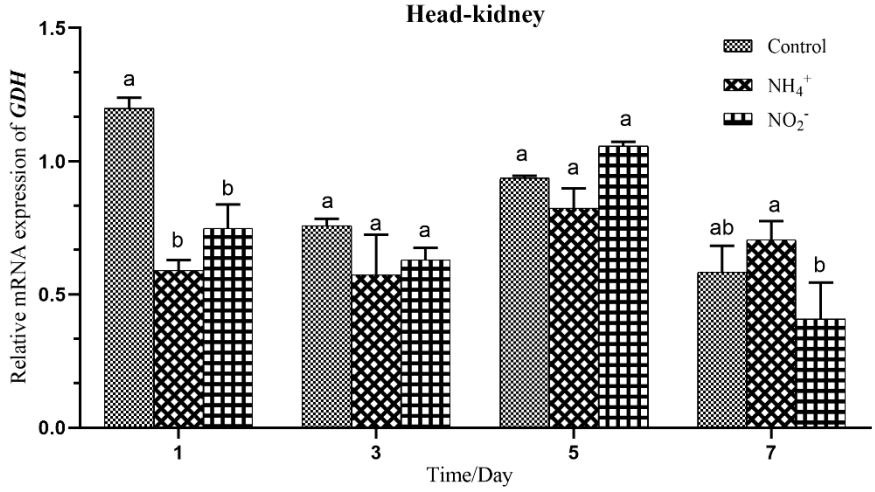
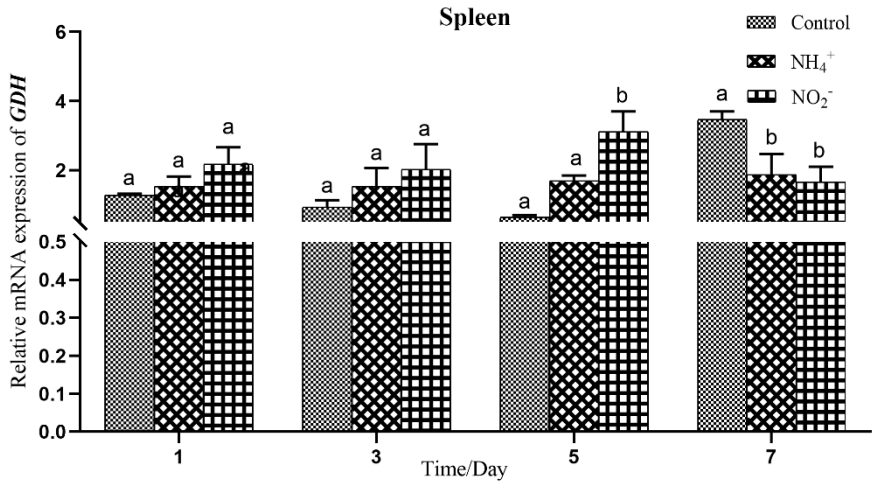


Figure 3 Glutamine synthetase (GS) gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where P < 0.05.

GDH expression under both NH_4^+-N and NO_2^--N stress was markedly downregulated in the head-kidney on day 1 ($P < 0.05$), then closely followed control levels. Under NH_4^+-N stress, it was upregulated in the gills; in the spleen and liver, it was upregulated at first, then downregulated by day 7. Under NO_2^--N stress, GDH was upregulated in the gill and muscle; in the spleen, expression initially increased, then at day 7 decreased. In the liver, it initially followed control levels for five days, but was then upregulated (**Figure 4**).


Muscle
Control
NH4+
NO2-
Relative mRNA expression of GDH
Time/Day
1
3
5
7


Head-kidney
Control
NH4+
NO2-
Relative mRNA expression of GDH
Time/Day
1
3
5
7


Spleen
Control
NH4+
NO2-
Relative mRNA expression of GDH
Time/Day
1
3
5
7

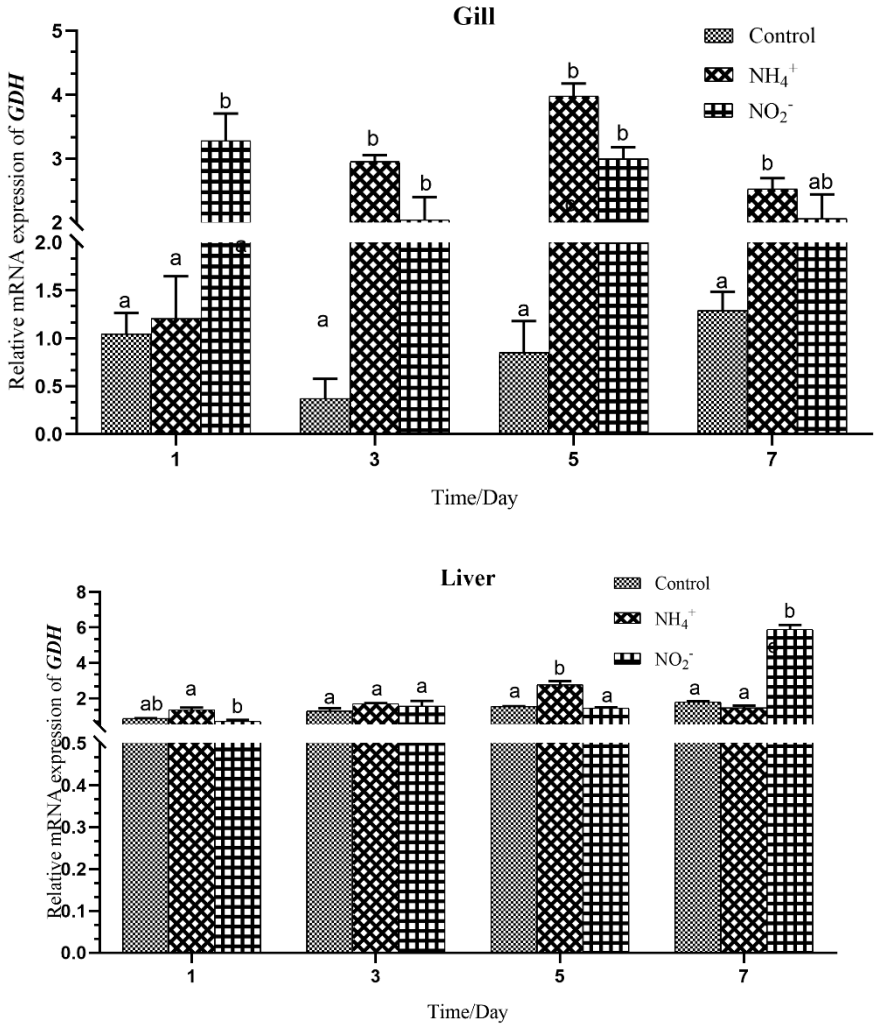


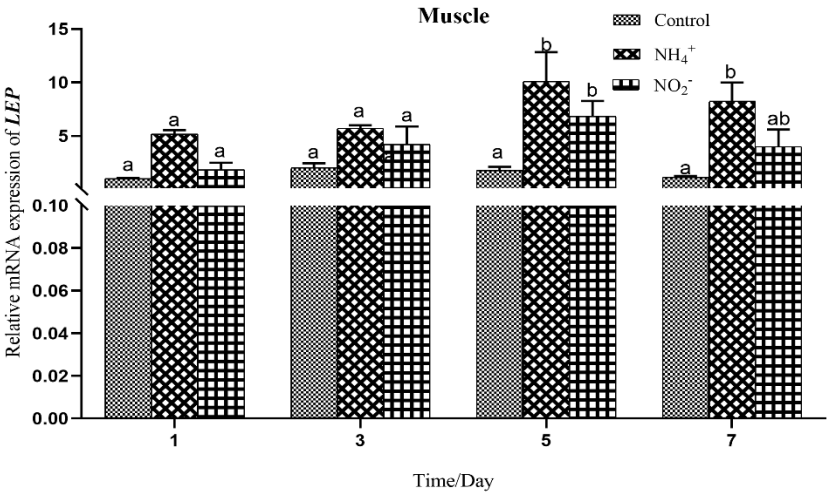
Figure 4 Glutamate dehydrogenase (GDH) gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.

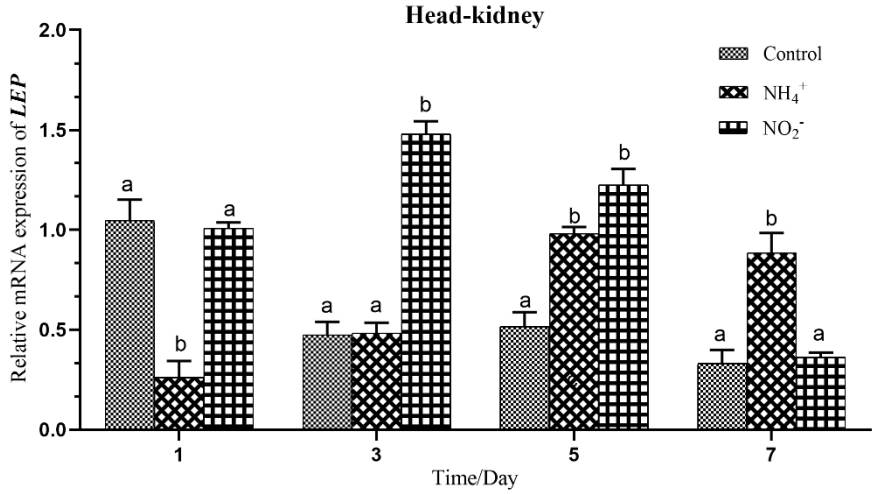
Other metabolism-related gene expression

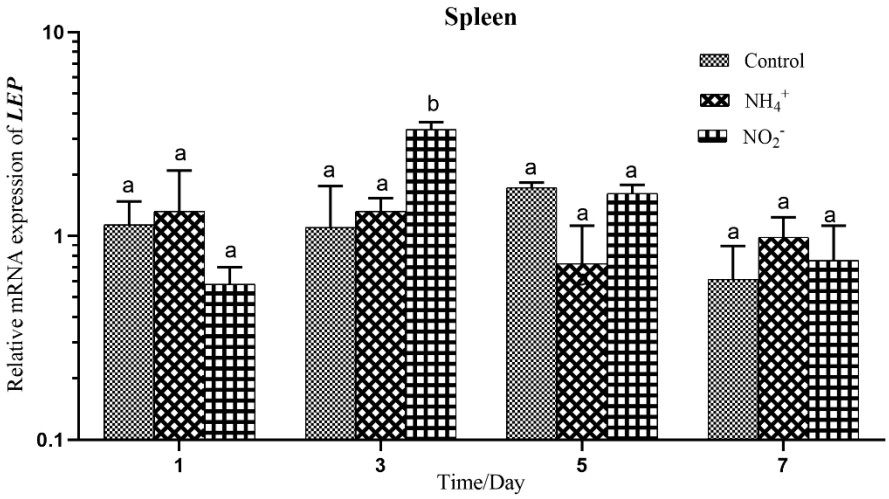
Under NH_4^+-N stress, LEP expression did not fluctuate significantly in the spleen or gill. On day 7, it was markedly upregulated in the head-kidney, liver, and muscle. Under NO_2^--N stress, LEP expression was initially unaffected, but then reached a peak value at day 3, followed by a decrease to control levels in the head-kidney and spleen; and always up in the muscle. LEP expression was always higher in the liver than in other tissues (**Figure 5**).

Under NH_4^+-N stress, the expression of IGF-1 was downregulated in the spleen, but did not change significantly in the head-kidney and muscle. In the liver, it initially rose, then fell at day 5. Under NO_2^--N stress, IGF-1 expression increased in the head-kidney, fell in gill, and was unchanged in the muscle. On day 3, it was also down-regulated in the spleen (**Figure 6**).

Under NH_4^+-N stress, CA expression was upregulated in the head-kidney, spleen, gill, and muscle. In the liver, it was initially upregulated, then returned to control levels. Under NO_2^--N stress, CA expression was upregulated in the spleen and gill. In the liver, it initially held steady, then decreased (**Figure 7**).


Muscle
Control
NH4+
NO2-
Relative mRNA expression of LEP
Time/Day


Head-kidney
Control
NH4+
NO2-
Relative mRNA expression of LEP
Time/Day


Spleen
Control
NH4+
NO2-
Relative mRNA expression of LEP
Time/Day

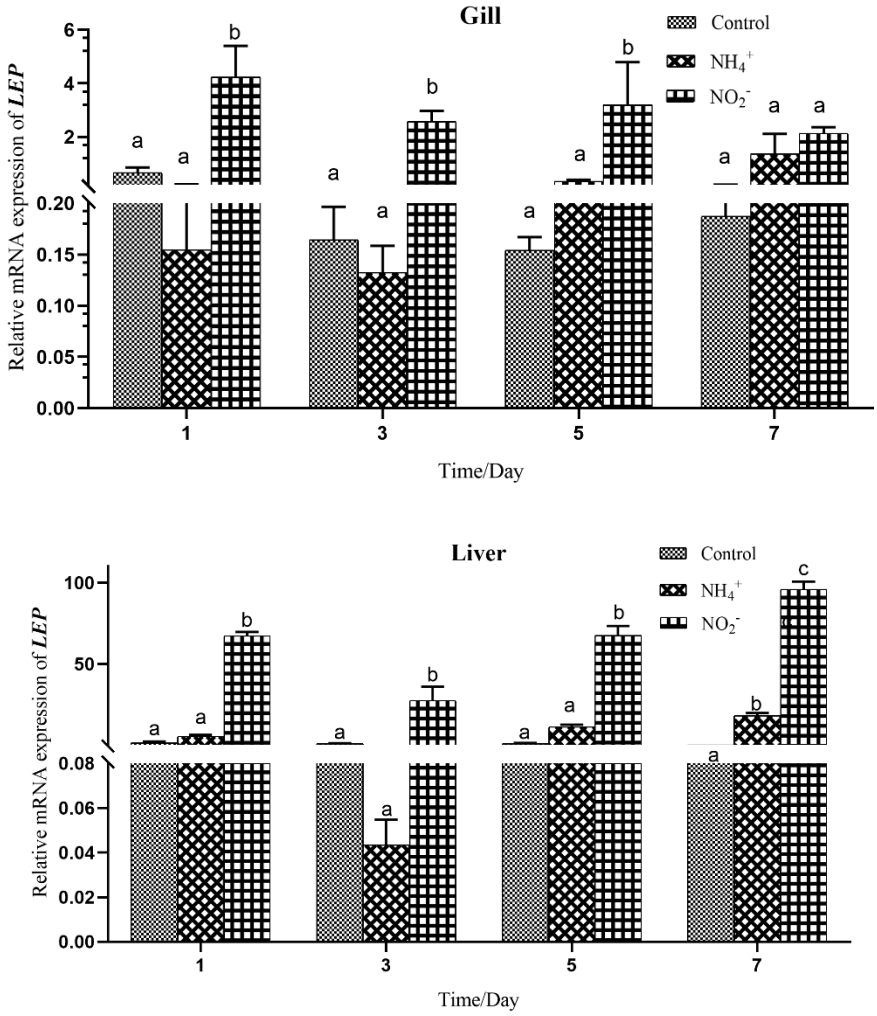
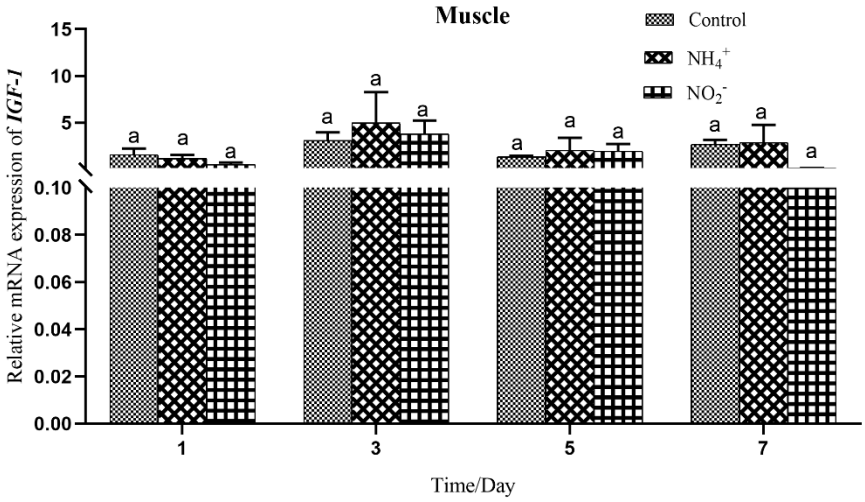
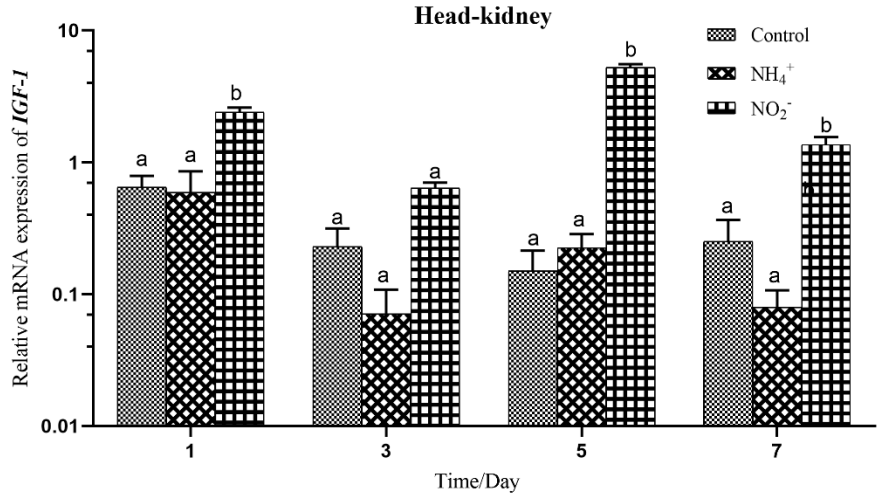
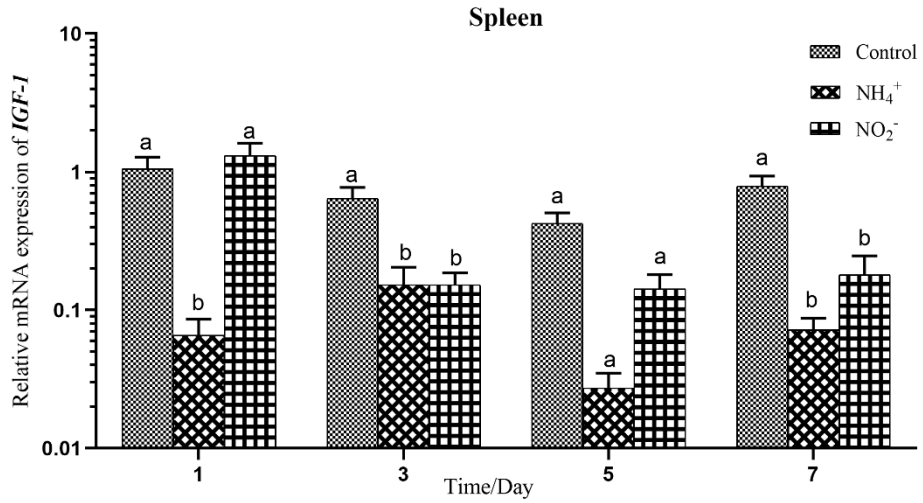


Figure 5 Leptin (LEP) gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.


Muscle
Control
NH4+
NO2-
Relative mRNA expression of IGF-1
15
10
5
0.10
0.08
0.06
0.04
0.02
0.00
a
1
3
5
7
Time/Day


Head-kidney
Control
NH4+
NO2-
Relative mRNA expression of IGF-1
10
1
0.1
0.01
a
b
1
3
5
7
Time/Day


Spleen
Control
NH4+
NO2-
Relative mRNA expression of IGF-1
10
1
0.1
0.01
a
b
1
3
5
7
Time/Day

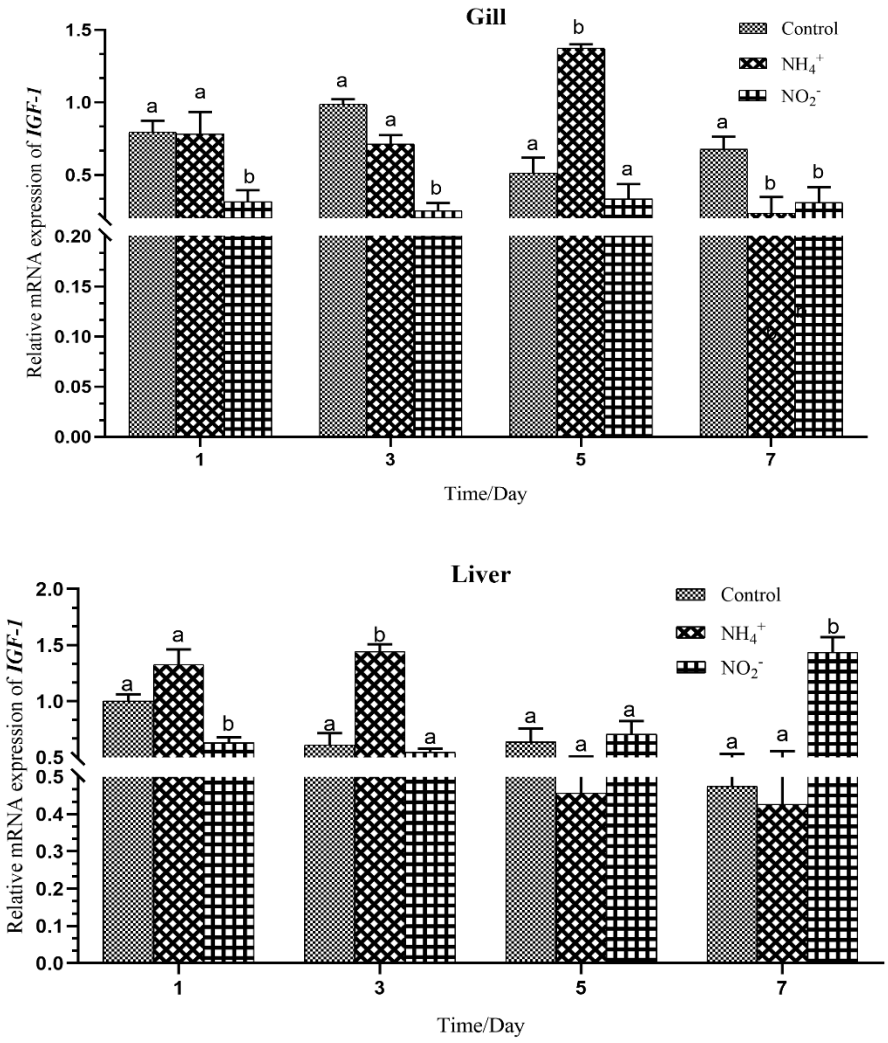
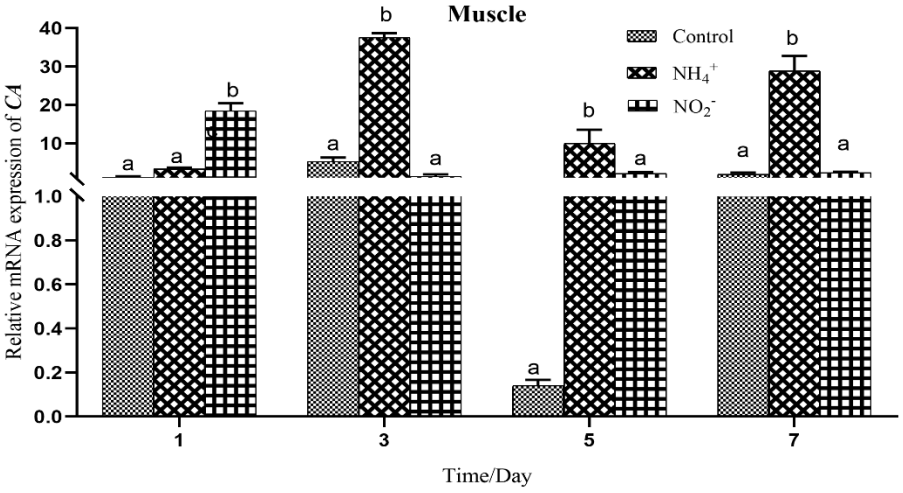
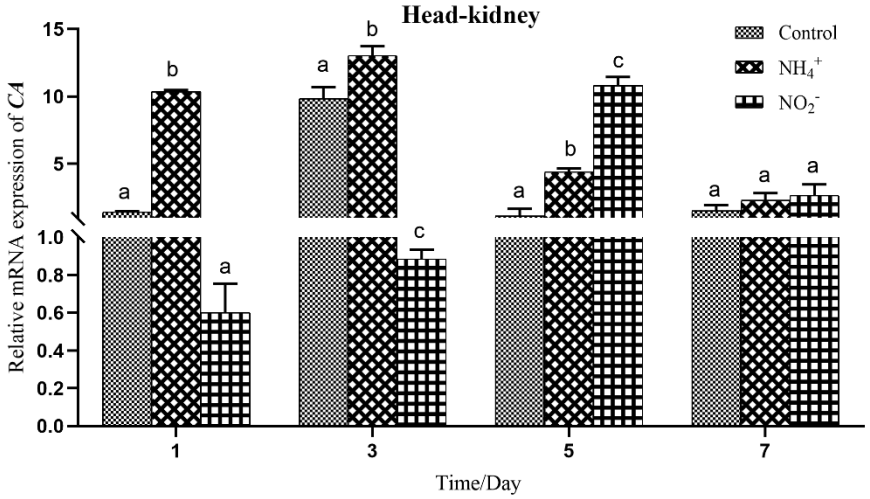
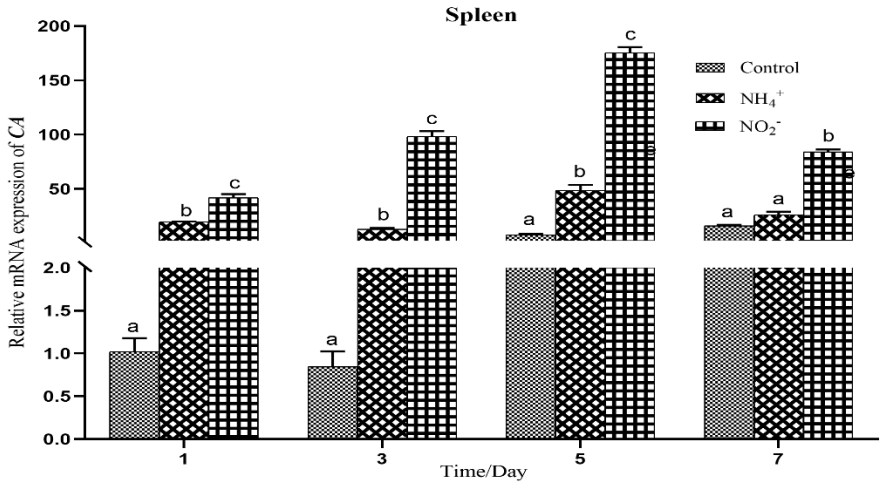


Figure 6 IGF-1 gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.


Muscle
Control
NH_4^+
NO_2^-
Relative mRNA expression of *CA*
Time/Day


Head-kidney
Control
NH_4^+
NO_2^-
Relative mRNA expression of *CA*
Time/Day


Spleen
Control
NH_4^+
NO_2^-
Relative mRNA expression of *CA*
Time/Day

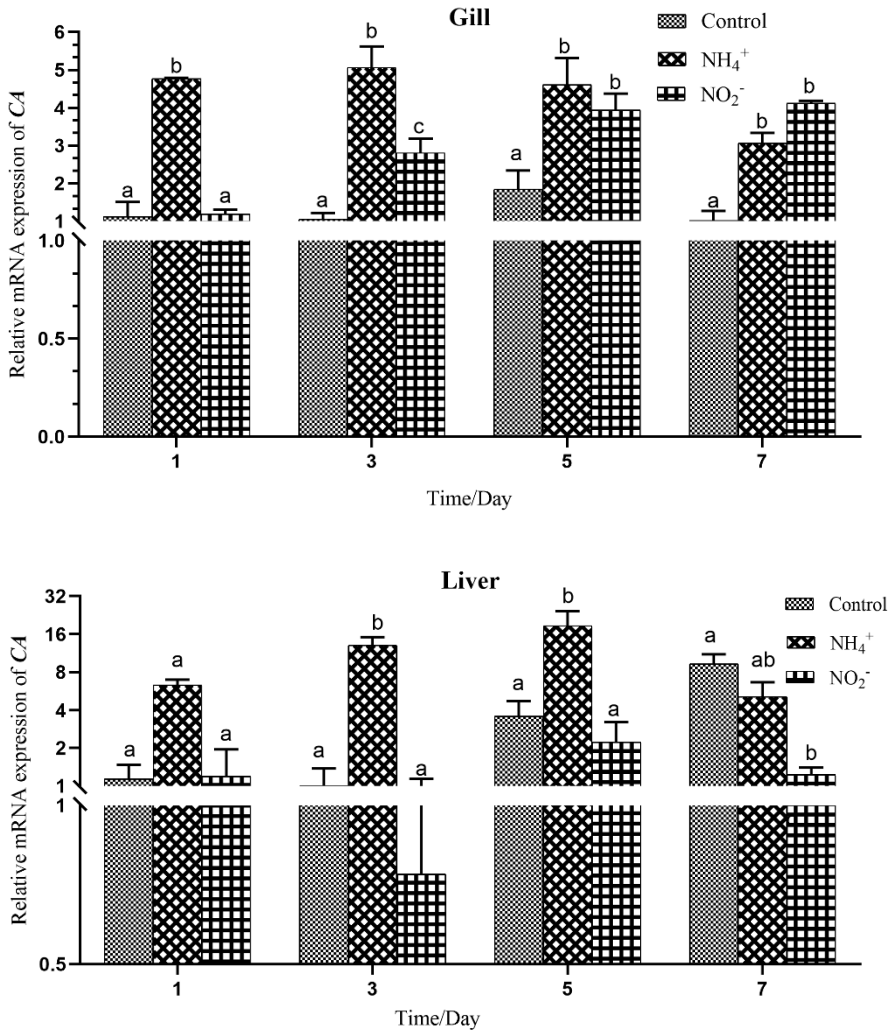


Figure 7 Carbonic anhydrase (CA) gene expression in muscle, head-kidney, spleen, gill, and liver of *L. japonicus*. Vertical bars represent the mean ± SE (n = 3). Lowercase letters a, b, and c indicate significant differences among treatments according to two-way ANOVA where $P < 0.05$.

Discussion

Ammonia and nitrite are vital environmental factors affecting aquatic animals, such as interfering with energy metabolism (Wang et al., 2012; Wang et al., 2014) and immune function (Li et al., 2015). A series of secondary diseases could accrue when pathogens invade fish in NH_4^+-N and NO_2^--N conditions. Many related studies focus on physiological and biochemical level, but we tend to analyze the functional gene as biomarker. Hence forecasts NH_4^+-N and NO_2^--N stress might influence the genes related to immune and metabolic of the *L. japonicus*.

Heat shock proteins (HSPs) are modulated in fish cells and tissues in response to a vast array of stressors (Basu et al., 2002; Iwama et al., 1998). Many authors have reported a significant increase in Hsp70 for numerous species exposed to ammonia and nitrite (Deane et al., 2007; Jensen et al., 1998). For example, the upregulation of Hsp70 and Hsp90 in the gills when juvenile turbot exposed to nitrite (Jia et al., 2016). Besides, other authors also found that the expression of Hsp70 and Hsp90 in grass carp was related to the level of aerobic metabolism (Cui et al., 2014). Nowadays, HSPs can serve as biomarkers for environmental stress in loggerhead turtle embryos (*Caretta caretta*) (Tedeschi et al., 2014), tilapia (*Oreochromis* spp.) (Pradeep et al., 2012), crucian carp (*Carassius auratus*) (An et al., 2014), and mud crabs (*Scylla paramamosain*) (Chenga et al., 2018).

Therefore, as sensitive indicators, HSPs can provide warnings of stress in fish. Although it is unclear how ammonia and nitrite promote Hsp70 and Hsp90 expression in fish tissues, HSPs could plausibly be induced via direct destruction of cell proteins, since damaged proteins can serve to

induce HSPs (Ananthan et al., 1986). Alternatively, nitrite exposure has been reported to cause methemoglobin formation in fish (Hilmy et al., 1987; Huertas et al., 2002; Woo & Chiu., 2010), resulting in reduced blood oxygen carrying capacity. Hypoxia stress has been shown to upregulate HSPs in animal tissues (Kobayashi et al., 1995; Mestril et al., 1994). In our study, Hsp70 and Hsp90 expression was upregulated in all five tissues in the initial stage under both treatments. The HSPs can utilize, bind and separate with energy produced by the hydrolysis of ATP, and finally complete the transportation, synthesis and remove the denatured proteins (Quanjie et al., 2015), which indicated that Hsp70 and Hsp90 could improve immunity by eliminating unfavorable proteins. GS and GDH are the key enzymes in nitrogen metabolism, involved in the metabolism of glutamate and proline, respectively. Thus, the two enzymes play a central role in maintaining the normal nitrogen level in organism (Phyllis et al., 2005; Chakrapani et al., 2017).

Glutamine accumulation has been observed in several organs in various species exposed to ammonia. Rainbow trout (*Onchorhynchus mykiss*) exposed to ammonia showed increased brain glutamine content and decreased glutamate content (Sanderson et al., 2010). In four-eyed sleeper fish (*Bostrychus sinensis*) (Peh et al., 2010) exposed to ammonia in a hyperosmotic environment, intestinal glutamine increased twofold, accompanied by a significant increase in the activity and mRNA abundance of intestinal GS. Since the magnitude of glutamine accumulation in the intestine was lower than in liver and muscle, which lacked changes in GDH activities, intestinal glutamate may have been shuttled to the liver and muscle to facilitate increased synthesis of glutamine there. Similar findings have been reported in giant mudskipper (*Periophthalmodon schlosseri*) (Chew & Ip., 2014).

In our study, GS and GDH expression was upregulated in the gills in both two stress groups. Notably, the level of GS expression was much higher than of GDH, which may mean that the detoxification effect of GS is more fundamental and faster than GDH under stress conditions. As we all know, GS and GDH cooperate to degrade inorganic nitrogen. Therefore, the expression of GS and GDH play a key role in maintaining normal nitrogen levels in an organism. Glutamine contributes to degrading toxicity of ammonia nitrogen in Gulf toadfish (*Opsanus beta*) (Veauvy et al., 2005), catfish (*Clarias gariepinus*) (Wee et al., 2007), rainbow trout (Wright et al., 2007), and swamp eel (*Monopterus albus*) (Tng et al., 2010), which has been proved. After synthesis of glutamine, it is discharged into the blood as a non-toxic ammonia metabolite from various tissues and finally reaches the liver. GDH and GS can also be considered indicators of ammonia and nitrite stress.

The effects of LEP on body weight regulation, fat metabolism, appetite regulation, bone remodeling, hematopoiesis, immune function, and reproduction have been showed in fine flounder (*Paralichthys adspersus*) (Fuentes et al., 2012) and Atlantic salmon (Murashita et al., 2011). Owing to its pleiotropic effects and potential for treating obesity, many leptin studies have been performed (Tinoco et al., 2012). Studies on striped bass (*Morone saxatilis*) (Won et al., 2012), rainbow trout (Laing et al., 2002), yellow catfish (*Pelteobagrus fulvidraco*) (Gong et al., 2013), and Chinese perch (*Siniperca chuatsi*) (He et al., 2013) all indicate that LEP is mainly expressed in the liver.

Furthermore, LEP expression in the brain of mandarin fish (*Siniperca chuatsi*) (Yuan et al., 2016) is high. Our findings indicated that LEP was widely expressed and especially abundant in the liver, consistent with many previous studies. In order to adapt to changes in the osmotic environment, fish need to expend energy to improve the metabolic level of the body (Bœuf & Payan, 2001), which has been mentioned in the study of *Lateolabrax maculatus* (Zhang et al., 2016). We speculate that this increased energy requirement necessitated the catabolism of liver glycogen, requiring upregulated LEP expression to contribute to glucose metabolism. As a product of the obese gene, leptin has a variety of physiological function including food intake and energy balance to counter the negative effects of NH_4^+-N and NO_2^--N stress.

IGF-1 is vital in regulating fish growth (Barzilai et al., 2009; Reinecke et al., 2005). In fish livers, IGF-1 mRNA is positively correlated with body weight gain in Nile tilapia (*Oreochromis niloticus*) (Cruz et al., 2006). Our results showed that the expression of IGF-1 mRNA was most abundant in the liver, but was the lowest of all the genes studied; Because of its small enrichment in tissues, IGF-1 was not strongly affected by ammonia and nitrite conditions. Although growth was not explicitly measured, the short-term stress experiments showed no obvious influence on body size. These results suggest that the liver of *L. japonicus* played a major role in the synthesis and secretion of IGF-1. Meanwhile, IGF-1 is not a suitable biological indicator for acute ammonia and nitrite stress.

The gills of some fish species are associated with osmoregulation, and upregulation of CA occurs under increased ammonia conditions (Nawata & Wood, 2009). The study suggested that CA activity provides important information about general patterns of gas exchange and acid-base balance in fish (Gilmour & Perry, 2009). We found that CA expression was upregulated in the gill and spleen under ammonia and nitrite stress. In fact, the head-kidney is a vital organ that partake of the ionic metabolism. So, we hypothesize that the head-kidney is involved in sea bass metabolism under stress. Study have shown that at low nitrogen levels, the time from nitrogen metabolism to carbon metabolism is reduced; at high nitrogen levels, this duration is increased (Yue, 2007). Therefore, along with CA's enrichment and upregulation in the spleen and gill under ammonia and nitrite stress, we predicted that CA would involvements in nitrogen metabolism, then participate in carbon metabolism latterly of high nitrite exposure. Meanwhile, increased CA expression should enhance acid-base balance regulation.

Sublethal environmental stress can cause stress response in fish. Although this is a protective response, continuous stress will inhibit the body's metabolism and immune defense function, leading to an increase sensitivity to various pathogens. In fact, NH_3-N causes excessive accumulation of glutamine (Glu), which causes the enlargement of glial cells in the fish (Wee et al., 2007). In addition, NH_4^+-N can cause depolarization on the surface of neurons. Both cumulative Glu and depolarized neurons activate N-methyl-D-aspartate receptor (NMDA receptor) on the surface of neurons. Overactivation of NMDA receptor promotes the synthesis of NO (Rao et al., 2002; Rodrigo et al., 2005) and activates Na^+-K^+-ATP enzyme. The activation of Na^+-K^+-ATP enzyme will accelerate energy consumption in the brain. The osmotic activity enzymes (Na^+-K^+-ATP and CA) play a central role in osmotic regulation by participating in the ions transport. Besides, excessive NO not only increases oxidative stress in the body, but also easily produces highly toxic nitrogen peroxide, which damages mitochondrial respiration, triggers ATP depletion and leads to cell death (Rodrigo et al., 2009; Olivier et al., 2012). Meanwhile, NO_2^--N can cause irreversible liver damage (Park et al., 2007), which leads to the decrease of the nitrite detoxification and affects normal liver function. Das (Das et al., 2004) and other findings that can prove this point. The upregulation of the liver gene related to metabolic (GS, GDH) and immune (Hsp70, Hsp90) lateral confirmed the liver's response. Besides, NO_2^--N also acts on vascular smooth muscle directly, resulting in the relaxation of vascular smooth muscle, vasodilation, drop of blood pressure and other adverse symptoms. Obviously, the expression of seven functional genes, including those related to immunity as well as metabolism capacity is diverse in tissues. *L. japonicus* respond to NH_4^+-N and NO_2^--N stress by a combination of these functional genes. The molecular mechanism of genes will be further explored by omics in the future.

Conclusions

Three primary, innovative and interesting results revealed the follow conclusions: (a) Expression varied across tissues, where HSPs related to immune function were highly expressed in the spleen and head-kidney under stress; (b) Gene GDH and GS related to nitrogen metabolism, were abundantly expressed in muscle; (c) Leptin (LEP) with energy metabolism function showed the highest upregulation in the liver eventually. *L. japonicus* maintain osmotic pressure and acid-base balance by improving immune function and enhancing metabolic activities, and various enrichment of seven functional proteins to alleviate NH_4^+-N and NO_2^--N stress. This work provides reliable bioindicators for sea bass to answer NH_4^+-N and NO_2^--N stress, which has a vital reference value.

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