Proteomic analysis of the effects of low salinity stress on liver of Naked carp (Gymnocyprinus przewalskii)

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Abstract. Naked carp is only distributed widely in Lake Qinghai. A unique feature of this species is its resistance to high salinity, which enables it to survive and adapt to the extreme environment of Lake Qinghai. Since Naked carp is an endangered species, we considered increasing its resources by reproduction and domestication measures in low salinity ponds. To investigate the molecular biological mechanism of the effects of low salinity stress on the liver of the cultured Naked carp, TMT markers combined with high-performance liquid chromatography-mass spectrometry (TMT-LC-MS/MS) were used to compare and analyze the difference in the expression of liver tissue protein between the cultured population (JH) and wild population (QH) in this study. Proteomic analysis results showed that 107 differential proteins were significantly expressed, and 102 of them were up-regulated. Functional and pathway enrichment analysis results found those differential proteins participate in molecular functions such as pyruvate kinase activity, alkali metal ion binding and potassium ion binding, and various biological processes of energy metabolism including glucose metabolism and lipid metabolism. Differential proteins were mainly concentrated in glycolysis and gluconeogenesis, retinol metabolism, linoleic acid metabolism, and steroid biosynthesis and lipid metabolism pathways.

1. Introduction
Naked carp (Gymnocypris przewalskii) is a plateau schizothorax fish. It is a unique native species only distributed in Lake Qinghai where has evolved for a long time during its formation and development[1]. They are also in the core position of the entire ecosystem of Lake Qinghai. Due to the disturbance factors of the natural environment and human activity, fish resources have been reduced sharply[2]. In recent years, the total amount of resources has been recovered by artificial breeding in low salinity ponds and releasing measures.

Lake Qinghai is located in the Qinghai Tibet Plateau with an altitude of 3200 meters, which is the largest brackish lake in China[3]. High salinity is one of the most important hydrological environmental characteristics of Qinghai Lake. Salinity is an important environmental factor for aquatic organisms. The changes in salinity can cause a series of physiological stress reactions, including energy metabolism, neuroendocrine, and ionization balance[4]. Therefore, the study of salinity to oxidative stress of fish and the physiological response of the fish body can help to understand the antioxidant mechanism of fish and guide the healthy farming of fish.
Proteomics is the study of the characteristics of protein from the entire level. Proteomic analysis can be used to understand the dynamics of metabolic regulation in animals[5]. Therefore, in this study, TMT markers combined with high-performance liquid chromatography-mass spectrometry (TMT-LC-MS/MS) tandem technology were used to compare and analyze the expression of differential proteins in the fish liver tissues, as well as key metabolic pathways and important differential proteins in cultured and wild Naked carp populations. It is helpful to understand the salinity adaptability and osmotic pressure regulation mechanism of Naked carp for the theoretical basis of brackish water and desalination culture.

2. Materials and Methods

2.1. Experimental fish
The breeding population (JH) was cultured with low salinity of 6 in the Qinghai Lake Naked Carp Rescue Center. The growth environment of the wild population (QH) in Lake Qinghai with high salinity of 15. Three experimental fish in each group were randomly selected and dissected in an ice tray. Their livers were taken and an appropriate number of tissues were weighed, then washed clean with PBS solution and stored at -80°C.

2.2. Protein extraction, TMT marker and quantitative analysis
The liver sample was added with the appropriate amount of protein lysate, and the supernatant was centrifuged to extract protein after boiling in a water bath. The protein was quantified by BCA method and stored at -80°C. One tube of TMT reagent was added to each 100μg polypeptide. Peptides are labelled step by step according to the instructions of the TMT kit[6]. Liquid chromatography-tandem mass spectrometry (LC-MS / MS) was used to analyze the proteins after chromatographic separation.

2.3. Bioinformatics analysis
Proteins with significant differences (P < 0.05) were examined for the difference between up-regulated and down-regulated protein expression levels greater than 1.5 times. BLAST2 GO software was used to annotate the GO function of the differential proteins. The number of GO classified differential proteins were calculated through the gene ontology database, and the identified differential proteins were compared and analyzed according to biological processes, molecular functions, and cell components. KEGG metabolic pathway annotation was performed for the differential proteins, and KO classification was performed to obtain the pathway information in which the protein sequences were involved.

3. Results

3.1. Identified protein by TMT
As shown in the Volcano Chart (Figure 1), A total of 7155 proteins were identified in this experiment, of which 6738 proteins had quantitative information, 288 were up-regulated and 129 were down-regulated. Compared with the QH population, there were 107 differentially expressed proteins, of which 102 were up-regulated and 5 remained unchanged.
3.2. Enrichment analysis of GO function of differentially expressed protein

Functional annotation and enrichment analysis of differentially expressed protein GO gene are shown in Figure 2. The results showed that they mainly included ADP metabolic process, Ribonucleoside diphosphate metabolic process, Purine nucleoside diphosphate metabolic process, Purine ribonucleoside diphosphate metabolic process, Nucleoside diphosphate metabolic process, Nucleotide phosphorylation, Nucleoside diphosphate phosphorylation, glycolytic process, ATP generation from ADP, Single-organism carbohydrate catabolic process, Carbohydrate catabolic process, Nicotinamide nucleotide metabolic process, Pyridine nucleotide metabolic process, Pyruvate metabolic process, Pyridine-containing compound metabolic process, Actin filament-based process and Purine nucleoside triphosphate metabolic process. It also participates in molecular functions such as pyruvate kinase activity, alkali metal ion binding and Potassium ion binding. Therefore, the differentially expressed proteins of Naked carp have various molecular functions and are involved in many biological processes under low salinity stress.
3.3. *Pathway enrichment analysis of differential proteins*

Significant enrichment (Figure 3) results showed that the two groups of differentially expressed proteins were mainly concentrated in 20 metabolic pathways. The most significant enrichment pathways are Glycolysis and gluconeogenesis, Retinol metabolism, Linoleic acid metabolism, Steroid biosynthesis and other important pathways. Our result suggested that naked carp re-adjusts and distributes the body’s osmotic pressure regulation ability, metabolism and energy to be adapted to the low salinity aquaculture water environment.

![Figure 3. Significant pathway enrichment analysis of differential proteins.](image)

4. Discussion

As the main energy metabolic organ of fish, the liver is responsible for the metabolism of matter and the supply of energy during the adaptation of environmental changes[7]. At the same time, a large number of enzymes and steroids will be involved in the metabolism of lipids and sugar by the liver[8]. Salinity is one of the most common environmental factors and has the deepest effect on the physiological function of fish. Our study showed that in a low-salt culture environment, compared with the wild population, the liver of Naked Carp had a very significant response in glucose metabolism and lipid metabolism. The GO study showed that the liver underwent significant changes during glucose metabolism. KEGG enrichment results also showed that compared with the QH population, the differences in glycolysis and gluconeogenesis in the JH population were extremely significant (P < 0.05). Glycolysis and gluconeogenesis are the two main processes of sugar metabolism in fish[9]. The energy consumption of fish liver in the process of osmotic pressure regulation basically comes from the metabolism of carbohydrates and other carbohydrates. Studies have shown that glycolytic metabolism shows certain adaptability after being adapted to the high salt environment[10]. The liver activates the gluconeogenic pathway that causes glucose levels in the blood to rise and preparing enough energy to cope with the effects of low salinity stress[11].

The adaptation of fish to low-salt stress causes a series of complex physiological, biochemical, morphological, and behavioural changes[12]. The changes of phospholipid fatty acid composition in the liver organ play an important role in the process of fish adaptation to environmental factors[13]. The results of our study showed that there were significant changes in lipid metabolism, such as Linoleic acid metabolism and steroid biosynthesis. The low-salt stress affected the signalling pathways of steroid biosynthesis, antigen processing and expression, digestion and absorption of fat and protein, glyceride, arachidonic acid and tyrosine metabolism significantly[14]. Under the salinity stress, fish can release
hormones such as cortisol to promote the production of glucose in fish[14],[15]. In addition, steroid hormones regulate the biosynthesis and metabolism of sugar, fats, and proteins, and have inhibitory immune responses, anti-inflammatory, anti-toxic, and anti-shock effects[16]. Therefore, the response mechanism of fish to low salt culture environment is the process of energy generation distribution and regulation of the body in the process of osmotic pressure ion regulation.

5. Conclusion
Fish are low-temperature aquatic vertebrates. Environmental stress can cause a series of changes in the physiological activities of fish, which require a lot of energy consumption. Salinity is one of the important environmental factors affecting the physiological state of aquatic animals, although naked carp have a strong permeation regulation ability to water salinity, but when the salinity of water changes, fish will readjust and distribute the body's osmotic pressure regulation ability, metabolism, and energy to adapt to salinity stress. The results show that in a low-salt culture environment, salinity stress has a significant effect on naked carp sugar metabolism and lipid metabolism.

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