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Metabolic response of Nile tilapia (*Oreochromis niloticus*) to acute and chronic hypoxia stress

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Abstract

Hypoxia is a critical issue in aquaculture especially in intensive aquaculture systems. Acute hypoxia stress with dissolved oxygen (DO) 0.7±0.1 mg/L for 6 h and chronic hypoxia stress with DO 1.1±0.1 mg/L for 4 weeks were used to investigate the response of nutritional metabolic pathways in Nile tilapia *Oreochromis niloticus*. Fish in the acute and chronic experiments had different adaptive mechanisms. Upon acute hypoxia stress, the contents of liver glycogen and muscle glycogen were significantly lower, but there was no significant difference in triglycerides (TG). The lactate dehydrogenase (LDH) activities increased after exposure to acute hypoxia stress. The mRNA expression of genes involved in glycolysis and glycogenolysis was significantly up-regulated by acute hypoxia stress. However, the response of fish to long-term hypoxia stress was different from acute hypoxia. Compared with the normoxia treatment, the crude fat in fish decreased in the hypoxia group and TG in the liver and muscle were significantly lower. Beta oxidation of the liver was enhanced in the hypoxia group, while the hepatic glycogen content increased in the hypoxia group. Transcriptomic analysis showed that the expression of genes related to carbohydrate synthesis and lipolysis increased in the hypoxia group, while genes related to carbohydrate catabolism and fat synthesis showed the opposite. This study indicates that fish could utilize carbohydrate as a main energy source during acute hypoxia stress, and metabolize more lipid during long-term hypoxia stress. A high carbohydrate content in the diet may help reduce negative effects from acute hypoxia stress, and an appropriate increase of fat content in the diet may benefit fish growth in a hypoxia environment, e.g., in high-density aquaculture ponds.

Keywords: hypoxia stress; *Oreochromis niloticus*; metabolic response; transcriptome

1. Introduction

Environmental hypoxia is a common challenge for many aquatic species as the aquatic environment has a wide range of temporal and spatial variations in oxygen levels compared to the terrestrial environment (Zhu C D, *et al.*, 2013). In recent years, natural and anthropogenic perturbation including high temperature, algal bloom, water pollution and the use of high-density aquaculture has caused local aquatic hypoxia in many parts of the world, which seriously restricts the distribution of aquatic species in nature and aquaculture development (Robertson C E *et al.*, 2014; Mahfouz M E, *et al.*, 2015). Hypoxia could affect the behavior, growth, food consumption and physiological state of fish. Juvenile spotted wolffish *Anarhichas minor* show lower weight gain and feed intake in a hypoxia habitat than the fish in the control and in the hyperoxic condition (Foss A, *et al.*, 2002). Hypoxia stress also has a negative effect on growth performance and immunity of Nile tilapia *Oreochromis niloticus* (Abdel-Tawwab M, *et al.*, 2015), and fish display sluggish behavior ($P < 0.05$) during hypoxia stress (0.8 and 0.3 mg/L) compared with the fish in the normoxic condition (Xu J, *et al.*, 2006).

A complex process of physiological and biochemical changes is involved in fish to cope with hypoxia stress (Terova G, *et al.*, 2008), including low metabolic rate, high ventilation and anaerobic respiration, and high haemoglobin O_2 affinity (Rahman M S, *et al.*, 2007). Except for physiological and biochemical alterations, hypoxia also affects nutrient metabolism (Polymeropoulos ET, *et al.*, 2017). In mammals, lactate and glucose production is enhanced by hypoxia stress in keratinocytes (Cuninghame S, *et al.*, 2017), and hypoxia can stimulate lipolysis and inhibit the uptake of free fatty acids (FFA) in adipocytes resulting in elevation of fatty acids in the plasma of obese mice (Yin J, *et al.*, 2009). The LDH activity in the gills of *Leiostomus xanthurus* increases significantly after 12 h hypoxia stress (0.8 mg/L) (Cooper R U, *et al.*, 2002). When amazon fish *Astronotus ocellatus* encountered a hypoxia stress, the blood glucose

concentrations significantly decreased (Bie M, 1998). In the liver of juvenile sea bass exposed to chronic hypoxia, glycogen content and lactate concentration decrease, and the expression of phosphoenolpyruvate carboxy kinase increases, indicating a stimulation of anaerobic glycolytic pathways (Cadiz L, 2017).

Although hypoxia could affect the metabolism of nutrients (Mahfouz M E, *et al.*, 2015), the differential metabolic responses to long-term and short-term hypoxia stress in fish are still little known. Exploring the mechanism of fish adapting to hypoxia will provide a better understanding of the nutrition demand and utilization under hypoxia stress, and the evolution in hypoxia adaption strategies in aquatic animals. Moreover, it will also bring new insights into the understanding of adaption to environmental stress in fish and provide a theoretical basis to improve aeration in modern aquaculture, especially for the development of high-density aquaculture. Transcriptome sequencing facilitates functional genomic studies, including global gene expression, novel gene discovery, and assembly of full-length genes (Gu J, *et al.*, 2015). This powerful new technology provides a platform to study the genetic and molecular response to a challenging environment for a species even without its genome reference database (Zhang G, 2016). Therefore, transcriptome was used in this study to obtain a comprehensive understanding on the metabolic response in tilapia at the transcriptomic level to explain physiological and biochemical observations.

The hypoxia tolerance of tilapia is higher than other farmed fish, and some tilapia can tolerate a hypoxia environment with dissolved oxygen below 0.5 mg/L for a short period (Teichert DR, *et al.*, 1997; Stickney RR, 2000; Lim CE, *et al.*, 2006), which makes it a suitable model species to study hypoxia stress in an aquatic habitat. Nile tilapia was chosen in this study not only because of its great hypoxia tolerance, but also the availability of its genome database (Chen XW, et al., 2017; Li HL, et al., 2017; Liu XY, et al., 2017). Therefore, the objective of this study was to explore the metabolic response of Nile tilapia to acute and chronic hypoxia stress and mechanism for fish to cope with acute

and long-time stress. Particularly, transcriptome was used to grasp a new understanding of nutrition metabolism in aquatic animals.

2. Materials and methods

2.1 Animals and feeding protocol

The experiment was carried out in indoor rectangular glass tanks in the Laboratory of Aquaculture Nutrition and Environmental Health, East China Normal University, Shanghai, China. The male Nile tilapia were obtained from a fish farm in Huadu District, Guangzhou. All the fish were stocked in 440 L tanks and fed with a commercial diet for 21 days to acclimate them to the culture condition before the trial started. During acclimation, the temperature of water was controlled at 28 ± 1 °C and pH at 7.3~7.9. Dissolved oxygen (DO) was maintained at 7 ± 0.5mg/L. All experiments were conducted under standard protocols for the Care and Use of Laboratory Animals at East China Normal University (F20140101).

2.2 Acute hypoxia trial

Thirty-Six fish (6.3±1.2 g) were randomly transferred into two groups of six tanks (100L), with 6 fish in each tank. Three tanks were maintained at normoxia and the other three were exposed to a hypoxia condition. The DO of hypoxic treatment was controlled at 0.7±0.1 mg/L through injection of nitrogen gas into the water. The DO of the control group was 7 ± 0.5mg/L and measured using a dissolved oxygen analyzer (Hach hq30d, America). The temperature of water was controlled at 28 ± 1 °C and pH at 7.3~7.9. After 6-hour hypoxia stress without feeding, three fish from each tank were taken to measure the oxygen consumption rate and fish behavior. Three fish from each group (one fish from each tank) was put in the ZebraCube behavior instrument for 30min one by one to detect the behavioral changes. The critical point between inact and smlct was set as 1mm/sec, while the point between smlct and larct was 5mm/sec. The behavioral changes of fish in 30min were recorded and analyzed. The other two fish of each tank were used

to measure the oxygen consumption rate, which was measured with a mitochondrial respiratory apparatus (Boshitong Co. Ltd., Shanghai) (28 ± 1 °C and DO at 7.0±0.5 mg/L). Other fish were anesthetized with 0.1 g/L ethyl 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, Sigma Aldrich Chemical Co., St. Louis, MO, USA) for weighing, counting and measuring. Three fish from each tank were sampled for liver, muscle and blood. The hemoglobinin was detected immediately after sample collection. Other blood samples were centrifuged at 3000 rpm for 5 min at 4 °C (Eppendorf, Germany), and then serum was removed and stored at −80 °C until use. The liver and muscle specimens were stored in liquid nitrogen at −80 °C soon after sampling. The liver samples were used for the detections of glycogen, TG, lactic acid (LA), enzyme activity of pyruvate kinase (PK), hexokinase (HK), LDH and mRNA expression.

Total RNA was extracted from the livers using a unizol reagent kit (Invitrogen) according to the manufacturer's protocol, whereas RNA quantity and quality were estimated by the absorbance at 260 and 280 nm with NanoDrop (Thermo, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. Total RNA was reversely transcribed using the PrimeScript^{TM} RT reagent kit (Takara, Shiga, Japan) for realtime quantitative (qRT-PCR) analysis. A pair of gene-specific primers of fructose1,6-bisphosphatase (*fbp*), glucose-6-phosphatase G-6-pase (*g6p*), phosphofructokinase (*pfk*), and *pk* was designed (SI Table 1). The amplifications were performed in a 96-well plate with a reaction volume of 20μL, containing 10μL SYBR Green Premix Ex Taq^{TM} (Takara), 0.4μL 10 mM gene-specific forward and reverse primers, 2μL diluted cDNA template (200 ng μL^{-1}) and 7.2μL H_2O. The PCR conditions were as follows: 95 °C for 30 s; 40 cycles of 94 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s and a 0.5 °C per 5s incremental increase from 60 to 95 °C. The data were analyzed using the CFX Manager TM software (version 1.0) (Bio-Rad, Hercules, CA, USA). Samples were run in triplicate and normalized to the control gene, β-actin. The cycle time (Ct) values of different tissues were calculated by the $2^{-\Delta\Delta Ct}$ comparative Ct method.

Muscle samples were used for the measurements of glycogen, TG and mRNA expression. Blood was used for hemoglobin analysis, and serum was for the detection of blood sugar, TG and LA.

2.3 Chronic hypoxia trial

After acclimation, another 180 fish (body weight 6.1 ± 1.3 g) were randomly transferred into six 440 L experimental tanks with 30 fish each. The temperature of water was controlled at 28 ± 1 °C and pH at 7.3~7.9. Three tanks were maintained at normoxia (6.5~7.5 DO mg /L) and the other three were exposed to a hypoxia condition (1.0~1.2 DO mg/L). The DO was detected in an hourly interval from 05:00 h to 23:00 h daily. Fish were fed to apparent satiation twice daily (09:00 and 17:00) with a commercial feed (5% crude lipid, 25% crude protein). 1h after feeding, the residue of diet was siphoned out, dried at 60℃and weighted. Feed intake was calculated by the weight of diet and the residue every day. Fish were taken out the tank, wiped with wet towel gently and weighted weekly. The oxygen consumption rate of fish in each tank were also measured once a week. After 28 days, fish in each tank was numbered and the survival rate was calculated by the formula: Survival rate (SR)= 100 ×(final fish number/ initial fish number). Fish were deprived of feed for 12 h and then behavioral changes and oxygen consumption rate were measured with the same protocol as in the acute hypoxia trial. Then fish were anesthetized with MS-222, and the serum and muscle were extracted and immediately stored in liquid nitrogen at −80 °C for further analysis. Part of the liver samples were fixed in paraformaldehyde (4%) for paraffin section and the rest of liver samples were also stored in liquid nitrogen.

2.4 Paraffin Section

The excised liver samples were fixed in paraformaldehyde (4%) for 24 h. Fixed liver were then dehydrated in ascending concentrations of alcohol and cleaned in xylol, followed by vacuum-embedding in paraffin. The embedded fish liver was sectioned with a rotary microtome at 5 μm. The tissue slices were stained with hematoxylin and eosin

(HE). The stained sections were analyzed using the BX51 system (OLYMPUS, Tokyo, Japan), and digital images were taken using Image-Pro plus 6.0.

2.5 Sample analysis

The proximate composition of the whole fish body was determined according to the standard methods of by the Association of Official Analytical Chemists. Moisture content was estimated by drying the samples to a constant weight at 105°C in a drying oven. The crude protein content was measured by the Kjeldahl method (8200, Kjeltec, Foss, Sweden). Crude lipid content was determined by the chloroform methanol method (Folch J, *et al.*, 1951). Blood glucose, TG, LA, glycogen, hemoglobin and enzyme activity of PK, HK, LDH and total lipase were detected with a biochemical indicators kit (Nanjing Jiancheng Bioengineering Institute) and the product number of these kits were shown in SI Table 2. A liquid scintillation counter was used for β-oxidation analysis. Wet liver tissues were weighed and homogenized (1:40, w/v) in an ice-cold 0.25 M-sucrose medium containing 2 mM-Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) and 10 mM-Tris-Cl at pH 7.4. Then the homogenate samples were used for immediate measurements of mitochondrial and peroxisomal [1-^{14}C] palmitate β-oxidation. The rate of total and peroxisomal palmitate oxidation was calculated from the radioactivity of the acid-soluble products. The β-oxidation ability of liver was calculated according to the degree of radiation (Pan H, *et al.*, 2017).

The liver transcriptome was analyzed by RNA-seq to obtain an overall view of the metabolic response to long-time hypoxia stress in the fish liver. Total RNA was extracted from the liver with three replicates using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (Takara, Japan). The quality and quantity of total RNA were assessed using a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, DE, USA). The RNA-seq transcriptome library was prepared following the TruSeq™ RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA from the liver. Messenger RNA was isolated

according to the poly A selection method using Oligo (dT) beads and then was fragmented using fragmentation buffer. Double-stranded cDNA of the two tissues was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). T4 DNA ligase buffer was used to end-repair the double-stranded cDNA. A single (A) was added using Klenow buffer. Adaptor-modified fragments were selected by gel-purification, and PCR amplification was performed for 15 cycles. After being quantified by TBS380, the paired-end RNA-seq sequencing library was sequenced using Illumina HiSeq 4000.

2.6 Calculations and statistical analysis

Each variable was analyzed using independent sample T test after being tested the normality by one-sample Kolmogorov-Smirnov test. The levels of statistical difference were set at $P < 0.01$ as extreme difference and $P < 0.05$ as significant difference. All analyses were performed using IBM SPSS Statistics 19 software (SPSS, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1 Effect of acute hypoxia on behavior, oxygen consumption rate and metabolism

Figure 1 shows the behavioral, physiological and biochemical responses of Nile tilapia after 6-h exposure to acute hypoxia stress. Fish under hypoxia stress swam on the water surface, showed slower movement ($P < 0.01$) and higher oxygen consumption ($P < 0.05$). Though the hemoglobin content of fish under hypoxia stress was higher than normoxia group, there was no significant difference (Fig. 1A, B, C). The mRNA expressions of *fbp* and *pk* were both up-regulated ($P < 0.05$) (Fig. 1D). Enzyme activity of LDH in the serum of tilapia after acute hypoxia challenge was higher than in the control group ($P < 0.05$) (Fig. 1E). Besides, acute hypoxia reduced serum glucose and tissue glycogen contents in tilapia ($P < 0.01$) (Fig. 1F, G), but increased serum LA ($P < 0.05$) (Fig. 1H). No significant difference was found in the TG content between the two groups ($P < 0.05$)

(Fig. 1I).

3.2 Effects of chronic hypoxia stress

3.2.1 Effects of chronic hypoxia stress on behavior, growth and oxygen consumption

Under chronic hypoxia, tilapia swam on the water surface, and showed slower movement than the control fish in the normoxia condition ($P < 0.05$, Fig. 2A). Chronic hypoxia also reduced feeding rate and weight gain of the fish, but not affected fish survival ($P < 0.05$, Fig. 2B, C, E). At the physiological level, oxygen consumption rate ($P < 0.01$) and hemoglobin content ($P < 0.05$, Fig. 2D, F) significantly decreased.

3.2.2 Effects of chronic hypoxia stress on metabolism

The mRNA expression of genes related to gluconeogenesis (*fbp*) and lipolysis (adipose triglyceride lipase, *atgl*; carnitine palmitoyltransferase 1, *cpt1*) were upregulated ($P < 0.05$), while the expression of genes related to glycolysis (glucokinase, *gk*) decreased ($P < 0.05$) in the liver under chronic hypoxia stress (Fig. 3A). Similar to the mRNA expression, the enzyme activities of HK and PK as the key enzymes of glycolysis also decreased, but the activity of total lipase increased in the liver ($P < 0.05$) (Fig. 3B). The hepatosomatic index (HSI) were not affected by hypoxia (Fig. 3C). The activity of key enzyme related to anaerobic glycolysis (LDH) also increased. Accordingly, the serum glucose, serum LA, liver glycogen and muscle glycogen were all increased by chronic hypoxia stress, but the TG content in serum and liver all decreased ($P < 0.05$, Fig. 3C, D, E, F). The whole body fat also decreased after long term stress ($P < 0.05$, Fig. 3G), but the β-oxidation of the liver increased ($P < 0.05$, Fig. 3H). The beta oxidation in peroxysome were not changed, while the reaction in mitochondria were increased by hypoxia stress.

3.2.3 Effects of chronic hypoxia stress on histological parameters

Visually, the total amount of the empty space where lipid droplets occurred in the liver of the hypoxia group was much lower than that in the control group (Fig. 4A, B). For Fig. 4A and B, we randomly chose three rectangles with the areas of 6174μm^2, and calculated

the area of the total empty space inside each rectangle. The total lipid area in the hypoxia groups was much less than that in nomoxia group by scientific statistics (Fig. 4C).

3.2.4 Transcriptomic analysis

In the whole liver transcriptomic analysis, over 14 000 different transcripts were analyzed and the expression of 451 genes was significantly different, of which 281 genes showed high expression, and the expression of other 170 genes were decreased. Thirty four percent of these 451 genes were related to nutrition and energy metabolism (Fig. 5 and Supplementary 1). Among all the changed pathways, there were 14 significantly changed pathways. Most of these 14 pathways were about metabolism, including tyrosine, glycine, serine, threonine, sulfur, fatty acid, linoleic acid, glyceride and vitamin metabolic pathways. The rest were mainly about Adenosine Monophosphate Activated Protein Kinase Pathway, Peroxisome Proliferator-activated Receptor signaling pathway, circadian rhythm and inflammatory factors. According to the analysis and classification of these 451 genes, a metabolic map was summarized, containing the key substrates, products and enzymes of nutrient metabolism (Fig. 6). Red boxes represent the genes of upregulated expression while the green boxes represent the genes with down-regulated expression. The expression of genes related to lipolysis: *atgl*, *ppar* (Peroxisome proliferator-activated receptor), *cpt1*, *cyp7a1* (Cholesterol 7-alpha hydroxylase) and glyconeogenesis: *gs* (glycogen synthase), *pepck* (Phosphoenolpyruvate carboxykinase), *aldocb* (aldolase C, fructose-bisphosphate, b) pathways increased, but those related to lipid synthesis: *fasn* (fatty acid synthase), *acaca* (acetyl-Coenzyme A carboxylase alpha) and glycolytic: *gck* (glucokinase hexokinase), *pdhb* (pyruvate dehydrogenase beta), *aclya* (ATP citrate lyase a) pathways reduced.

4. Discussion

Either acute or chronic hypoxia stress may disturb physiological homeostasis, and adversely affect fish growth and health (Aboagye D L, *et al.*, 2017). In a hypoxia

environment, various behavioral responses could occur in fish to better adapt to the environment. Swimming to water surface is a typical adaptation in most fish species to a hypoxia habitat (Lewis W M, 1970). In the present study, tilapia swam to the water surface to gulp air when they were under acute and chronic hypoxia stress. The gulping behavior is a clear indication that fish are suffocated and need more oxygen for respiration (Kramer D L, *et al.*, 1982). We also found that the swim of tilapia slowed down under hypoxia stress, which is similar to the reports in Antarctic clam *Laternula elliptica* (Morley S A, *et al.*, 2007) and Atlantic cod *Gadus morhua* (Herbert N A, *et al.*, 2005). It is an adaptive behavior of fish to reduce energy and oxygen consumption under hypoxia stress.

Under acute hypoxia stress, fish usually reduce oxygen consumption by slowing down movement and improving oxygen-carrying capacity through the increase of red blood cells and hemoglobin concentration (Cossins AR, *et al.*, 2005; Roesner *et al.*, 2006; Xia *et al.*, 2016). In the present study, fish frequently swam to the water surface and also increased hemoglobin concentration during acute stress. However, we found that the oxygen consumption rate increased after fish were moved from a hypoxia water to a normoxia water, which is possibly an oxygen compensatory effect when a plenty of oxygen is available during the period of recovery.

In both vertebrates and invertebrates, glycogen metabolism is the main pathway of energy acquisition, especially in an unstable environment (Bacca H, *et al.*, 2005; Karlsson J, *et al.*, 1979; Oliveira G T, *et al.*, 2004). In the present study, the glycogen content decreased and the activity of glycolysis increased, suggesting that carbohydrate metabolism also plays an important role of energy supply when fish cope with acute hypoxia stress. When dissolved oxygen in water cannot satisfy the oxygen requirement for aerobic glycolysis, the normal physiological function and metabolic rate cannot be maintained (Richards J G, *et al.*, 2011). It has long been known that hypoxia is associated with the activation of anaerobic metabolism, and anaerobic glycolysis would meet the

high energy requirement of animals during hypoxia stress (Bie M, *et al*., 1998; Speers-Roesch B, *et al*., 2010; Bartrons R, *et al*., 2007). Due to the low ATP yield of anaerobic glycolysis, the substrates such as glycogen and glucose will be substantially consumed, leading to accumulation of lactate (Richards J G, *et al*., 2011; Genz J, *et al*., 2013). In the present study, we found a reduction of both glucose and glycogen but an increase of LA in the serum of tilapia.

Long-term hypoxia decreased the feeding rate and weight gain of tilapia. Appetite suppression is an early response among all other responses under hypoxia stress in fish (Pichavant et al., 2001; Bernier N J, *et al*., 2005; Bernier N J, *et al*., 2012.). Growth is usually related to the amount of feed intake in fish, which is reflected by weight gain reduction due to low feed intake caused by hypoxia in post-smolt Atlantic salmon (Mette R, 2012), big sea bass *Micropterus salmoides*, common carp *Cyprinus carpio*, turbot *Scophthalmus maximus* and the silver salmon *Oncorhynchus kisutch* (Pichavant, K, *et al*., 2001; Ruyet, *et al*., 2003; Brett J R, *et al*., 1981). However, the survival rate was not affected by dissolved oxygen in this study. This might be because of the high tolerance of low oxygen in tilapia. The hypothetical explanation is that the hypoxic responses including morphological, respiratory and metabolic adaptations in tilapia might result in unaffected survival in this study. In contrast to acute hypoxia, both hemoglobin concentration and oxygen consumption decreased in tilapia after chronic stress in this study, maybe the Nile tilapia has adapted to the chronic hypoxia stress. The reduction of oxygen consumption may be related to low hemoglobin concentration and slow movement of fish under chronic hypoxia stress.

In aquatic animals, metabolic reprogramming occurs to adapt to available energy reserve during environmental hypoxia (Gracey A Y, *et al*., 2011). Glycolysis, especially anaerobic glycolysis may be the main energy source under acute hypoxia stress according to the results in this study. However, the metabolic reprogramming in chronic hypoxia stress showed an opposite pattern in this study. The decrease of *gk* mRNA

expression and low activities of HK and PK indicate the overall reduction of glycolysis under chronic hypoxia, while the activation of anaerobic metabolism is associated with chronic stress as detected in acute stress. Many animals exposed to a prolonged hypoxia would inhibit glycolytic activation to prevent from metabolic acidity (Sidell B D, 1983; Van Waarde A, *et al*., 1983; Affonso E G, *et al*., 2002). In this study, the high glycogen content in the liver and muscle indicated the enhancement of gluconeogenesis, which was similar with the finding in a goby *Gillichthys mirabilis* (Gracey A Y, *et al*., 2011). The enhancement of gluconeogenesis in chronic hypoxia is probably due to lactate oxidation through the change of metabolic fuel preference to the use of lactate as a gluconeogenic substrate (Omlin T, et al., 2010). Another possible reason is the replenishment of glucose or glycogen that was over-consumed by anaerobic glycolysis to maintain energy balance in tilapia. With the reduction of glycolysis, the prolonged hypoxia increased *atgl* and *cpt1* expression, indicating the enhancement of lipolysis. Because the ATGL catalyzes the initial step in triglyceride hydrolysis and plays a central role in the degradation of lipid droplets known as adiposomes (Zimmermann R, *et al*., 2004; Smirnova E, *et al*., 2006). The CPT1 is the rate-limiting enzyme for β-oxidation of long-chain fatty acids (Harpaz S, 2005).

The total lipase activity and TG content in the serum and liver are indicators of lipid catabolism (Zhao W M, *et al*., 2007). Similar to the results of this study, the pathway analysis generated by transcriptome reveals that the pathways related to triglyceride hydrolysis are upregulated while the pathways related to triglyceride synthesis are downregulated in the hypoxia-tolerant burrow-dwelling goby *Gillichthys mirabilis* under hypoxia stress (Gracey A Y, *et al*., 2011). Similar to this study, lipid metabolism also plays an important role in the adaptation to severe chronic hypoxia in *Drosophila*, and the expression of Brummer lipase and the fly ortholog of *atgl* are elevated (Azad P, *et al*., 2009). This is in contrast to the hypoxia effects in mammals where a rise in free fatty acids and glycerol occurs due to the adrenergic stimulation of phospholipid and lipolysis,

and the inhibition of β-oxidation by the absence of oxygen (Moore K H, 1985; Yin J, *et al*., 2009; Gimm T, *et al*., 2010). However, the available results of fish response to hypoxia in the current literature are controversial. Some indicate a decrease of lipolytic activity responding to hypoxia, while others show the opposite (Raaij M T M V, *et al*., 1994; Raaij M T M V, *et al*., 1996; Haman F, *et al*., 1997). The conflicting results may be related to species difference, ability of hypoxia tolerance and exposure time to hypoxia. The glycolysis was suppressed and lipolysis was elevated in tilapia exposed to a prolonged hypoxia in this study, indicating that the energy demand during long-time stress is mainly derived from lipid catabolism.

These findings led us to search for further evidence on the changes in lipid metabolism, in the hypoxia-challenged fish. The β-oxidation, especially the mitochondria was stimulated in the liver of tilapia. Moreover, the crude fat of whole fish reduced after 4-week hypoxia stress. Histological sections of the liver tissue from the normoxic control and fish exposed to the prolonged hypoxia reveal morphological differences in structure. The liver from the control contained larger vacuoles, but the size vacuoles in the hypoxia fish was shrunk, an evidence of lipid degradation. These histological data support the enzyme and metabolic evidence and indicate that the lipolysis has replaced glycolysis to supply energy under a chronic hypoxia challenge in tilapia.

Transcriptome analysis was carried out to further confirm the results of metabolic responses to long-time hypoxia stress. The transcriptomic analysis shows that carbohydrate synthesis and lipolysis were enhanced, while carbohydrate catabolism and lipid synthesis were reduced. There is no obvious difference in amino acid metabolism, demonstrating the importance of lipid catabolism under hypoxia stress. These results are also supported by the biochemical data. In conclusions, lipid was the main source of energy during long-time hypoxia stress.

In conclusion, hypoxia stress would induce the fish to swim to the water surface, and slow down fish swimming, reduce feed intake and growth, and change the pattern of

metabolism after a prolonged hypoxia challenge. The energy metabolism was also affected by the dissolved oxygen concentration and stress duration. Carbohydrate metabolism, especially anaerobic metabolism, plays an important role of energy supply when fish cope with acute hypoxia stress. But lipolysis would replace glycolysis to supply energy under a chronic hypoxia challenge in tilapia.

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Figure legends

Figure1. Tilapia responses to acute hypoxia stress. A. Behavioral change to acute hypoxia stress (Inact: inactive, Smlct: moderately active, Larct: hyperactive). B. Oxygen consumption. C. Hemoglobin content. D. Relative mRNA expression of *fbp* and *pk*. E. The activity of LDH (lactic dehydrogenase) in serum. F. The content of glucose in serum. G. The glycogen content in liver and muscle. H. The LA content in serum. I. TG (triglyceride) content in serum, liver and muscle. Note: "*" means significant difference ($P < 0.05$), "**" means extremely significant difference ($P < 0.01$), MEAN±SE, N=3 for Panel A and N=6 for Panel B-I.

Figure 2. Growth performance of tilapia under chronic hypoxia. A. Behavioral change (Inact: inactive, Smlct: moderately active, Larct: hyperactive). B. The survival rate of Nile tilapia. C. Feeding rate over time. D. Oxygen consumption rate. E. Weight gain. F. Hemoglobin content. Note: "*" means significant difference ($P < 0.05$), "**" means extremely significant difference ($P < 0.01$), MEAN±SE, N=3 for Panel A and N=6 for Panel B-F.

Figure 3. Metabolic responses of tilapia to long-term hypoxia stress. A. The mRNA relative expression for glycometabolism of glucokinase (*gk*) and *fructose1,6-bisphosphatase (fbp)* and lipid metabolism of *atgl* (adipose trigtyceride lipase) and *cpt1* (carnitine palmitoyltransferase 1). B. PK, HK, LDH and total lipase activity in liver. C. Hepatosomatic index of fish. Hepatosomatic index (HSI)= 100 ×(liver weight / body weight). D. The content of glucose in serum. E. The glycogen content in liver and muscle. F. The LA content in serum. G. TG content in serum, liver and muscle. H. The crude fat of whole fish (dry sample). I. The β-oxidation in liver. Note: "*" means significant difference ($P < 0.05$), "**" means extremely significant difference ($P < 0.01$),

MEAN±SE, N=6.

Figure 4. The paraffin section of liver in the control and hypoxia (Arrows refer to fat).

Figure 5. The classification of the 451 significantly down-regulated and up-regulated genes detected by transcriptome.

Figure 6. The changes of metabolic pathways after chronic hypoxia stress. Red boxes are genes with increased expression and green ones are genes with decreased expression.

Supplementary 1. The heat map of the genes related with nutrition and energy metabolism. Different columns mean different groups (C1, C2, C3: The three parallel of normoxia group. T1, T2, T3: The three parallel of hypoxia group.), and different rows mean different genes. Red color represents up-regulated genes, and green represents down-regulated genes.

Figure 1.

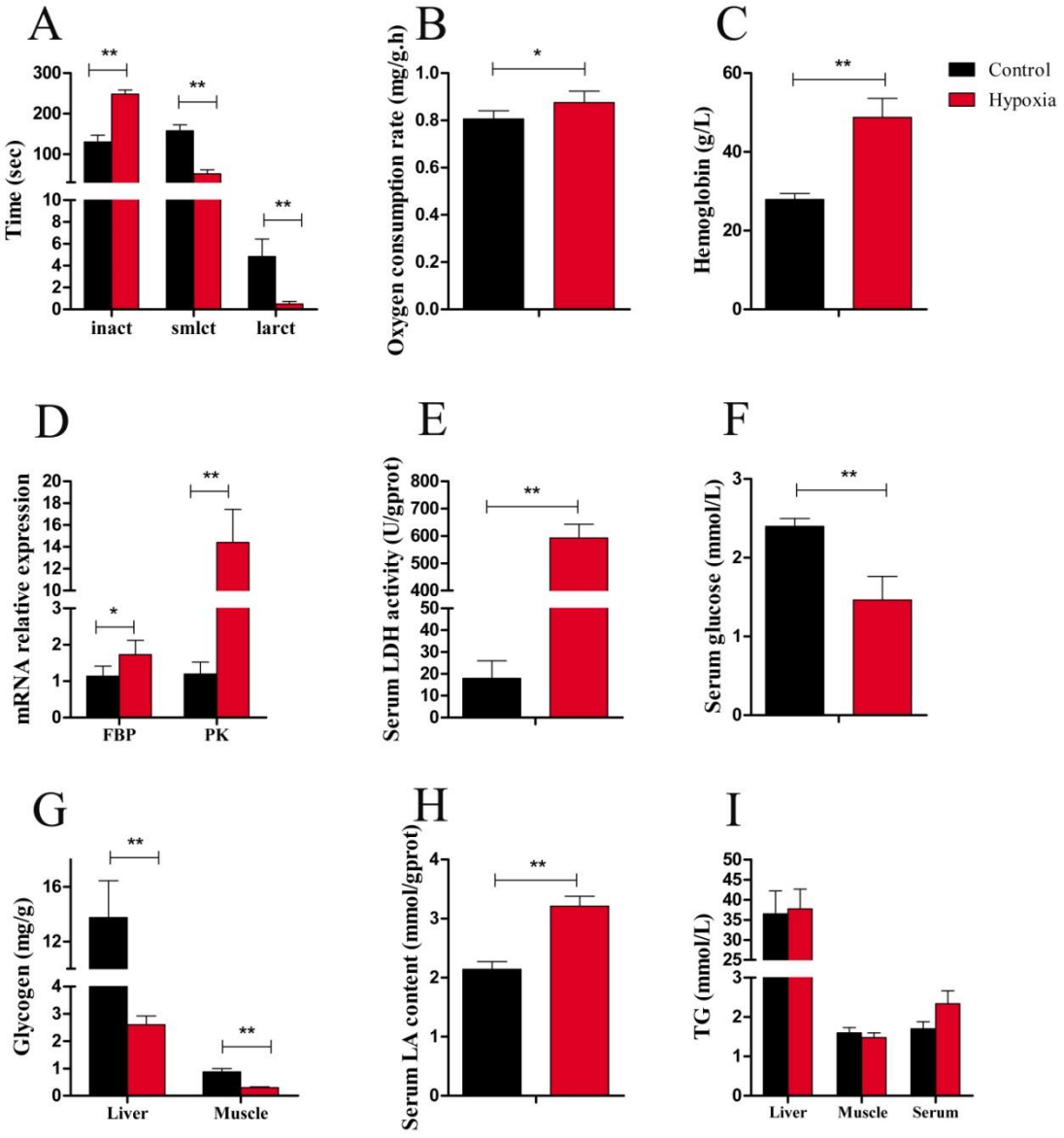


Figure 2.

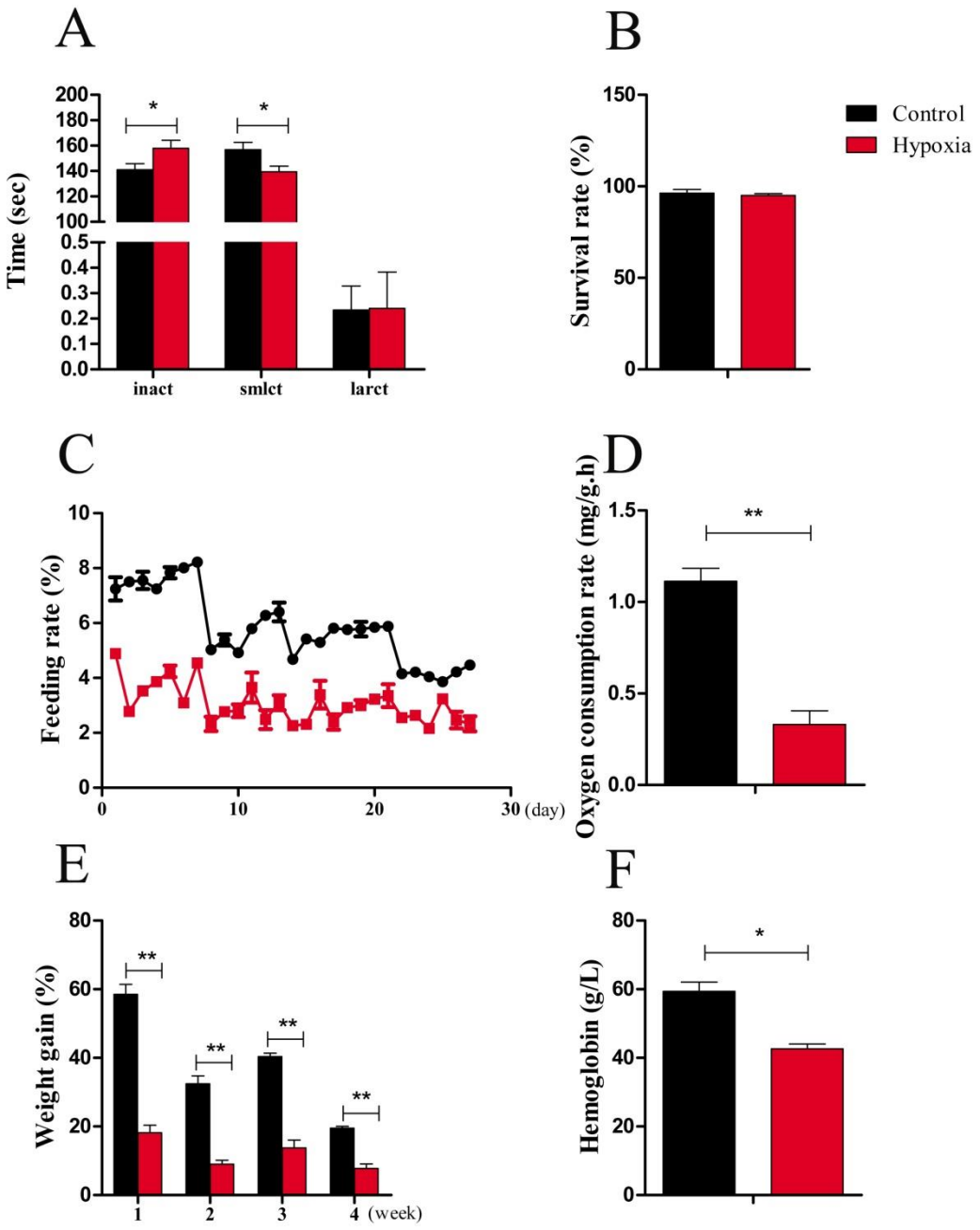
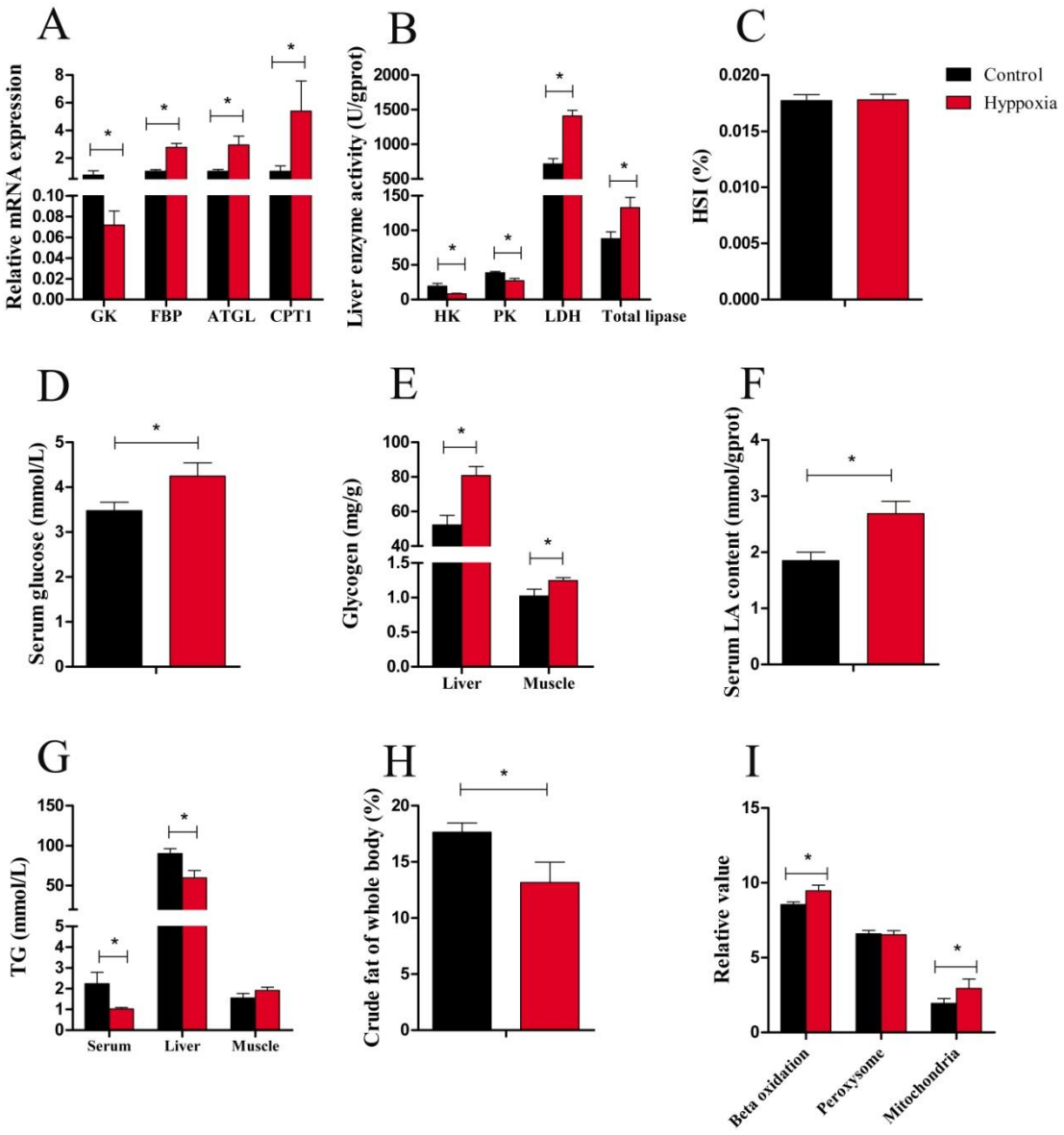


Figure 3.



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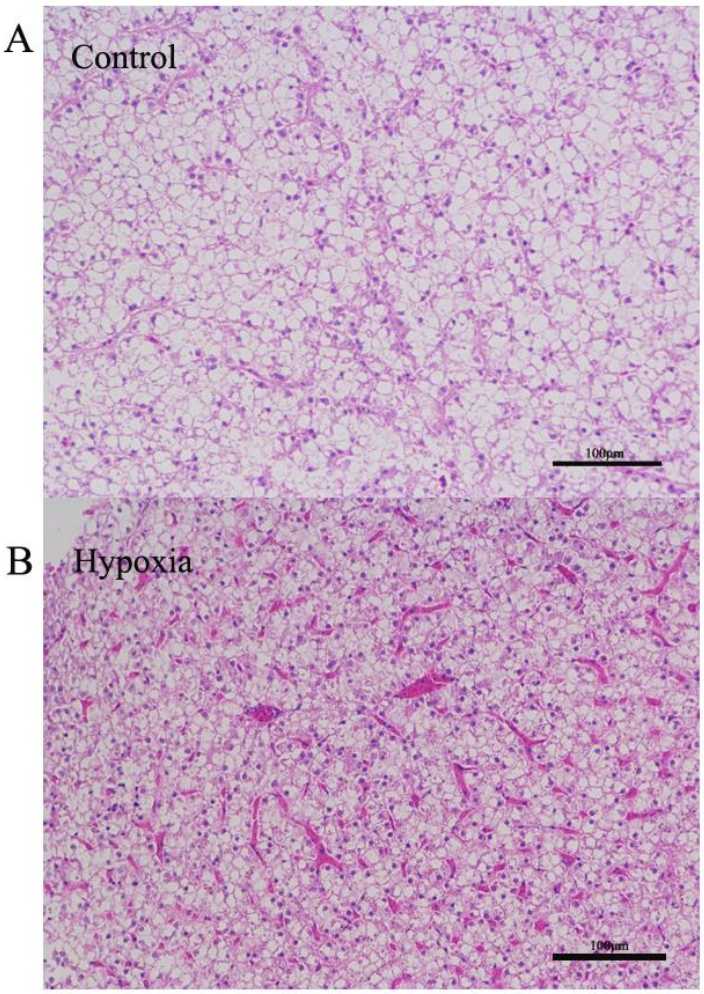


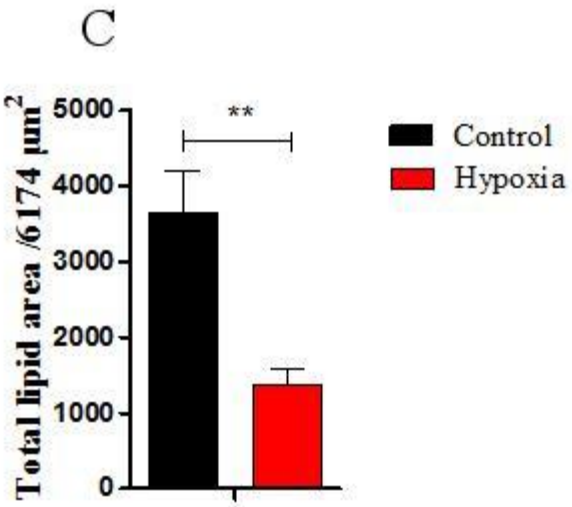
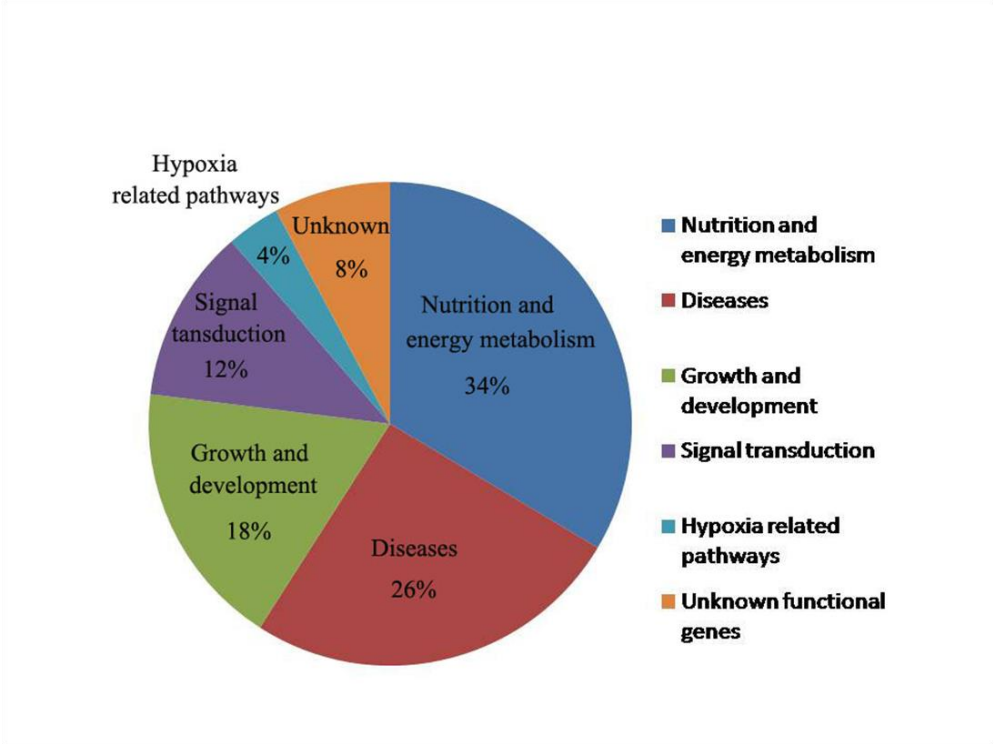


Figure 5.



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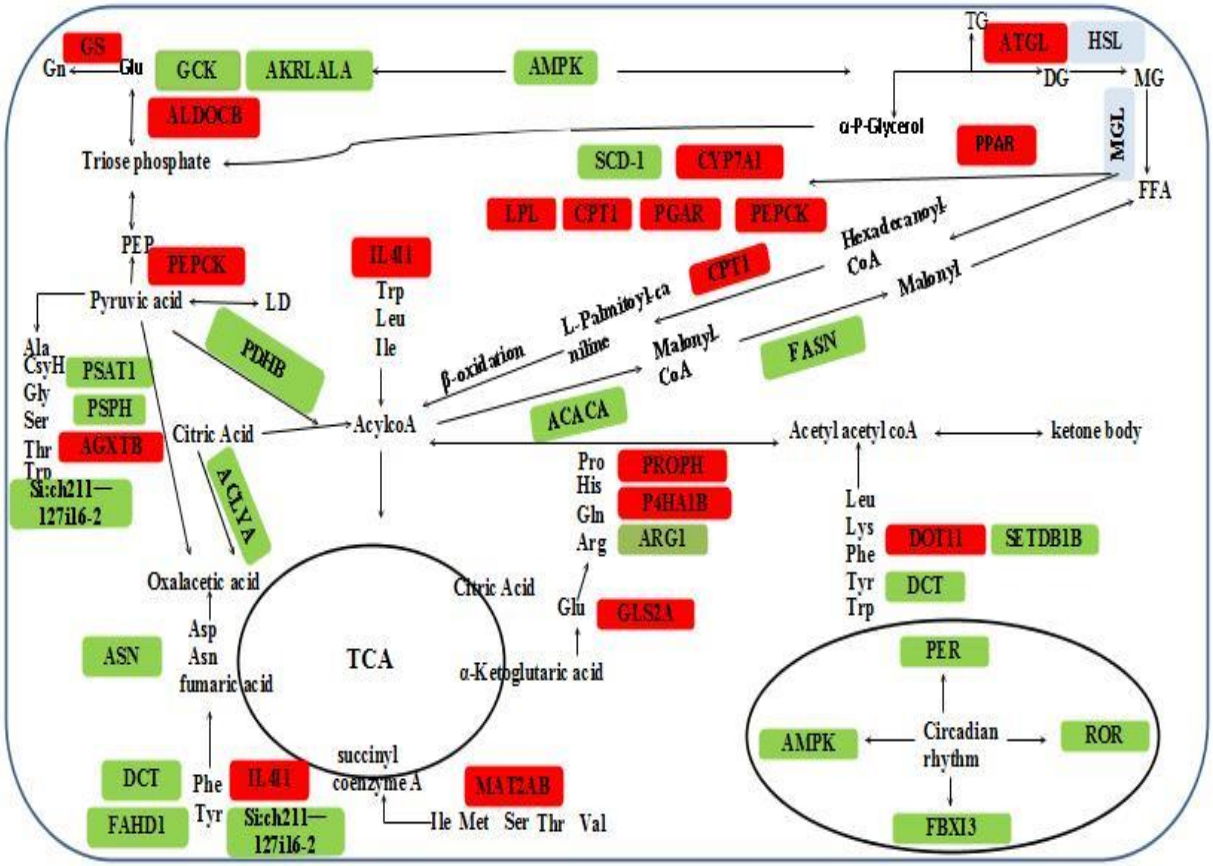

SI Table.1. The primers used for Q-PCR analysis

Accession No.	Item	Gene name	Sequence(5'-3')
KJ123689	Forward primer	EF1a	ATCAAGAAGATCGGCTACAACCCT
	Reverse primer		ATCCCTTGAACCAGCTCATCTTGT
XM_003449650.2	Forward primer	FBPase	ACCGGACAATAGCGGAAAATACA
	Reverse primer		TGGCGAATATTGTTCCTATGGAGA
XM_003448671.2	Forward primer	G6Pase	AGACCTTATTGGTGGGTTCACGA
	Reverse primer		CTGAAGGACTTCCTGGTCCAGTTT
XM_003441476.2	Forward primer	PFK	AACCTGTGTGTGATTGGAGGTGAT
	Reverse primer		CGTGATCTTACCGGCTTTAACAAG
XM_005472623.1	Forward primer	PK	CAGCATAATCTGCACCATCGGT
	Reverse primer		ATGAGAGAAGTTAAGACGGGCGA
FJ601660	Forward primer	HSL	AACCTGGATGTCCATTTCTGGAAG
	Reverse primer		TCGGTTTACCTTGACTTGAGTGGA
XM_003440346	Forward primer	ATGL	AAAACGTCCTGGTGACCCAGT
	Reverse primer		TAGGAGGAATGATGCCACAGTACA
XM_005478351.1	Forward primer	MGL	ACATCGTCAACGCAGACGGATT
	Reverse primer		CACAATGTTCCCCAGCTCCAT
KF871430	Forward primer	PPARα	CTGATAAAGCTTCGGGCTTCCA
	Reverse primer		CGCTCACACTTATCATACTCCAGCT
XM_003451020.2	Forward primer	GK	GACATGAGGACATTGACAAGGGAA
	Reverse primer		CTTGATGGCGTCTCTGAGTAAACC
XM_003440552	Forward primer	CPT1	TTTCCAGGCCTCCTTACCCA
	Reverse primer		TTGTACTGCTCATTGTCCAGCAGA

SI Table.2. The biochemical indicators kit of the experiment

The kit	The number
The indicators kit of LDH	A020-2
The indicators kit of LA	A019-2
The indicators kit of glycogen	A043
The indicators kit of TG	A110-1
The indicators kit of Glu	F006
The indicators kit of GPT	C009-2
The indicators kit of G0T	C010-2
The indicators kit of Total lipase	A067
The indicators kit of HB	C021
The indicators kit of PK	A076-1
The indicators kit of HK	A077-1

Highlights

- The investigation of the metabolism under hypoxia stress in this manuscript would provide some new ideas for hypoxia stress mitigation in aquaculture.
- The lipid was the main energy source during long-time hypoxia stress, which indicated that an appropriate increase of fat content in the diet may benefit fish growth in a hypoxia environment, e.g., in high-density aquaculture ponds.
- We found that carbohydrate metabolism is the main energy source in tilapia during acute hypoxia stress, which gave us an enlightenment that high dietary carbohydrate might help reduce negative effects of acute stress in aquaculture.