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Dietary thiamine modulates carbohydrate metabolism, antioxidant status, and alleviates hypoxia stress in oriental river prawn *Macrobrachium nipponense* (de Haan)

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ABSTRACT

Hypoxia is one of the challenges in prawns aquaculture. However, the role of thiamine, which is a coenzyme in carbohydrate metabolism with antioxidant properties, in reducing hypoxia in prawns aquaculture is currently unknown. We investigated the effects of thiamine on antioxidant status, carbohydrate metabolism and acute hypoxia in oriental river prawn, *Macrobrachium nipponense*. One thousand eight hundred prawns (0.123 ± 0.003 g) were fed five diets (60 prawns each tank, six replicates per diet) supplemented with graded thiamine levels (5.69, 70.70, 133.67, 268.33 and 532.00 mg/kg dry mater) for eight weeks and then exposed to hypoxia stress for 12 h followed by reoxyegnation for 12 h. The results showed that, under normoxia, prawns fed the 133.67 or 268.33 mg/kg thiamine diet had significantly lower glucose 6-phosphatedehydrogenase, succinate dehydrogenase and phosphoenolpyruvate carboxykinase activities than those fed the other diets. Moreover, total antioxidant capacity (T-AOC) increased significantly when prawns were fed the 133.67 mg/kg thiamine diet. Superoxide dismutase (SOD) activity and malonaldehyde (MDA) content also increased significantly when prawns were fed the 268.33 or 532.00 mg/kg thiamine diet under hypoxia. And the significantly increased SOD activity and MDA level also observed in prawns fed 532.00 mg/kg thiamine under reoxygenation. Under normoxia, prawns fed the 70.70 or 133.67 mg/kg thiamine diet decreased the mRNA expressions of AMP-activated protein kinase-alpha (*AMPK-α*), pyruvate dehydrogenase-E1-α subunit (*PDH-E1-α*) and hypoxia-inducible factor-1s (*HIF-1α*, *HIF-1β*), but increased the mRNA expressions of phosphofructokinase (*PFK*) significantly. After 12 h of hypoxia, the energy metabolism related genes (*AMPK-β, AMPK-γ, PFK, PDH-E1-α*), hypoxia-inducible factor related genes (*HIF-1α*, *HIF-1β*) and thiamine transporter gene (*SLC19A2*) were up-regulated significantly in prawns fed the 133.67 or 268.33 mg/kg thiamine diets. After 12 h of reoxygenation, prawns fed the 133.67 or 268.33 mg/kg diet significantly decreased the SOD activity, MDA level and *SLC19A2* mRNA expression compared with other diets. The optimum thiamine was 161.20 mg/kg for minimum MDA content and 143.17 mg/kg for maximum T-AOC activity based on cubic regression analysis. In summary, supplementing 143.17 to 161.20 mg/kg thiamine in the diets for *M. nipponense* improves the antioxidant capacity under normoxia and reduces the oxidative damage under hypoxia stress.

1. Introduction

Hypoxia is a condition that occurs when the amount of dissolved oxygen (DO) in the water gets depleted less than 2 to 3 mg/L. DO in water is easily affected by sudden weather conditions such as temperature, air pressure and precipitation [1]. In recent years, global warming and climate change caused by the greenhouse effect have made the water body become hypoxic more frequently [2]. In addition, high-density farming and artificial diet feeding also affect the amount of dissolved oxygen in the water [3].

Hypoxia causes energy loss, reactive oxygen species (ROS)

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Abbreviations

18S rRNA	The 18S ribosomal RNA
AMPK-α	The AMP-activated protein kinase-alpha
AMPK-β	The AMP-activated protein kinase-beta
AMPK-γ	The AMP-activated protein kinase-gamma
G6PDH	glucose 6-phosphatedehydrogenase
H12h	12 h of hypoxia
HIF-1α	hypoxia-inducible factor-1α
HIF-1β	hypoxia-inducible factor-1β
HIFs	Hypoxia-inducible factors
MDA	malonaldehyde
NADPH	nicotinamide adenine dinucleotide phosphate
N0h	0 h of normoxia
PDC	Pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase
PDH-E1-α	pyruvate dehydrogenase-E1-α subunit
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
R12h	12 h of reoxygenation
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SOD	superoxide dismutase
SLC19A2	solute carrier family 19 members A2
SLC19A3	solute carrier family 19 members A3
T-AOC	total antioxidant capacity
TCA	tricarboxylic acid

accumulation, resulting in metabolic disorders and oxidative stress in organisms [4]. Aquatic organisms are more prone to hypoxia problems due to the nature of their environment compared with terrestrial animals [5]. Hypoxia disturbs energy metabolism, which activates anaerobic glycolysis, and generates lactate with insufficient ATP energy [6]. Therefore, to meet energy requirement under hypoxia, animals undoubtedly need more glucose for anaerobic glycolysis [7]. As hypoxia progresses, the limited oxygen availability causes increased ROS production due to an ineffective electron transfer in the mitochondria especially when flux is not attenuated through the tricarboxylic acid (TCA) cycle [8]. Reoxygenation causes oxygen to react rapidly with the electrons accumulated in the mitochondria leading to the rise in ROS production [9]. Increased ROS production triggers apoptosis, which causes severe oxidative mitochondrial damage [10]. Therefore, reducing carbohydrate flow into the TCA and increasing antioxidant capacity of cells is important for the survival of organisms during hypoxia.

Pyruvate dehydrogenase complex (PDC) is one the main pathways involved in carbohydrate metabolism in animal cells, which links glycolysis and the TCA cycle by catalyzing the conversion of pyruvate into acetyl-CoA [11]. The PDC is regulatory by phosphorylation-dephosphorylation of pyruvate dehydrogenase (PDH). The PDH activities require thiamine as a cofactor [12]. Thiamine, also known as vitamin B1, becomes a metabolically active coenzyme when esterified with two phosphates, yielding thiamine pyrophosphate [12]. In addition to PDH, oxoglutarate dehydrogenase and transketolase require thiamine as a cofactor [13]. Thus, thiamine is essential for normal carbohydrate metabolism in most organisms.

Most animals have limited ability to synthesize and store thiamine for maintaining its intracellular concentration and thus require supplementation in diets [14]. The need for thiamine supplementation increases in animals under stress due to intracellular disturbance, which interferes with cellular carbohydrate metabolism and energy production [15]. Therefore, animals have developed regulatory and adaptive mechanisms in order to maintain intracellular thiamine concentration. Indeed, solute carrier family 19 members A2 (SLC19A2) and A3 (SLC19A3), encoding thiamine transporter 1 and thiamine transporter 2, respectively, have been reported as regulators for transporting thiamine [16,17]. Currently, studies on the two genes are mainly focused on mammals, possibly related to typical diseases such as cancer, diabetes and Alzheimer's disease, which cause thiamine deficiency [18,19]. The role of thiamine in alleviating hypoxia remains largely unexplored in cultured prawns.

Thiamine may play a significant role in alleviating hypoxia stress in animals either directly or indirectly. Directly, thiamine alleviates hypoxia because it reacts with ROS [20]. The NH_2 group in thiamine structure reacts with ROS to generate dehydrothiamine, thereby eliminating the harmful effects caused by ROS to cells [21]. Indirectly, thiamine reduces hypoxia stress through its regulators such as SLC19A3, which directly bind with hypoxia-inducible factor-1α (HIF-1α), consequently up-regulates the SLC19A3 expression during hypoxia stress [15]. Hypoxia-inducible factors (HIFs) are a highly conserved transcription factors family that acts as principal regulators of oxygen homeostasis and adaptive response to hypoxia by reducing energy expenditure and increasing energy synthesis [22,23]. The regulated target genes of HIFs are involved in glycolysis, gluconeogenesis, glucose transport, antioxidant and oxygen transport in crustaceans [24,25]. Accordingly, HIF-1 enhances the tolerance of crustaceans to hypoxia stress and increases their survival in the wild environment [26]. Therefore, supplementing the diet for aquatic animals with thiamine may be useful to alleviate hypoxia stress. However, studies on the role of thiamine on hypoxia stress in cultured prawns are currently limited.

Oriental river prawn (*Macrobrachium nipponense*) is the only native species among large-scale farmed freshwater prawns in China with high economic benefits and huge consumer market demand [27]. *M. nipponense* has high oxygen consumption and is very sensitive to a low oxygen environment compared with other crustaceans [28]. Therefore, this study investigated the role of thiamine supplementation in alleviating hypoxia stress in *M. nipponense*. The ability of thiamine to attenuate hypoxic stress was assessed by determining growth performance, antioxidant capacity, and the mRNA expression of genes involved in thiamine transportation, hypoxia-inducible factor, carbohydrate and energy metabolism. The knowledge generated is important for formulation of *M. nipponense* diet for sustainable aquaculture.

2. Materials and methods

2.1. The source of prawns and experimental diets

Juvenile *M. nipponense* used in the experiment were purchased from Zhejiang Wuyue Agriculture Co., Ltd. Before the feeding trial, the prawns were fed a control diet for two weeks to acclimate to the experimental conditions. A basal diet was formulated by using casein and fishmeal as protein source, fish oil and soybean oil as lipid source, and corn starch as carbohydrate source. Five levels of thiamine (0, 60, 120, 240 and 480 mg/kg thiamine) were designed, and the final measured values were 5.69 (control group), 70.70, 133.67, 268.33 and 532.00 mg/kg thiamine, respectively, as determined by the high-performance liquid chromatography (HPLC) method detailed by Sander et al. [29]. The thiamine used in the experiment was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (CAS Number: 532-43-4, Shanghai, China). The concentrations of thiamine used in this study were based on previous studies on crustaceans [30,31]. The formulation and composition analysis (dry matter percentage) of the experimental diets are shown in Table 1. The diets manufacturing process involved accurate weighing of each ingredient according to the

Table 1
Ingredients and proximate compositions of the experimental diets (%, dry weight).

Ingredients	0	60	120	240	480
Fishmeal	28	28	28	28	28
Casein	20	20	20	20	20
Gelatin	5	5	5	5	5
Corn starch	26	26	26	26	26
Soybean oil	3	3	3	3	3
Fish oil	1.5	1.5	1.5	1.5	1.5
Vitamin premix[a]	2	2	2	2	2
Thiamine	0	0.006	0.012	0.024	0.048
Mineral premix[b]	3	3	3	3	3
Cholesterol	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.5	0.5	0.5	0.5	0.5
Soybean lecithin	0.5	0.5	0.5	0.5	0.5
Cellulose	8	7.994	7.988	7.976	7.952
Sodium carboxymethylcellulose	2	2	2	2	2
Total	100	100	100	100	100
Proximate composition					
Dry matter	94.15	94.01	94.15	94.13	94.29
Crude protein	40.18	39.85	39.81	39.75	39.78
Crude lipid	7.73	7.68	7.56	7.60	7.60
Crude ash	7.28	7.27	7.27	7.22	7.16
Thiamine (mg/kg)	5.69	70.70	133.67	268.33	532.00

[a] Vitamin mixture (g kg^{-1} mixture): vitamin A 1.29, vitamin C 57.5 mg, α-tocopherol acetate 20, vitamin D3 0.63, vitamin K 1.8, vitamin B_2 2.63, vitamin B_6 1, vitamin B_{12} 0.15, niacin 5, folic acid 0.19, inositol 60, biotin 0.75, calcium pantothenic 5.

[b] Mineral mixture (mg g^{-1} mixture): KCL 28; $MgSO4{\cdot}7H_2O$ 100; NaH_2PO_4 215; KH_2PO_4 100; $Ca(H_2PO_4){\cdot}2H_2O$ 265; $CaCO_3$ 105; $C_6H_{10}CaO_6{\cdot}5H_2O$ 165; $FeC_6H_5O_7{\cdot}5H_2O$ 12; $ZnSO_4{\cdot}7H_2O$ 4.76; $MnSO_4{\cdot}H_2O$ 1.07; $AlCL_3{\cdot}6H_2O$ 0.15; $CuCl_2{\cdot}2H_2O$ 2.4; $CoCl_2{\cdot}6H_2O$ 1.4; KI 0.23; α-cellulose 0.043.

formulation. Soybean lecithin and cholesterol were dissolved in the mixture of fish oil and soybean oil to make a homogenous mixture. Each diet was mixed separately and pellets (diameter, 1.5 mm) were produced by using an F-26 twin-screw extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China). The diets were dried at 40 °C for 24 h. The dried diets were sealed in labeled plastic bags and stored at − 20 °C until needed for feeding *M. nipponense*.

2.2. Feeding trial under normoxia

After the acclimatization period, 1800 visually healthy *M. nipponense* with an average body weight of 0.123 ± 0.003 g were randomly distributed into 30 tanks (60 prawns per tank, each with a capacity of 250 L). Each of the five dietary treatments had six replicates and *M. nipponense* in each tank were fed twice daily to apparent visual satiation at 07:00 and 16:00 h for eight weeks. Nylon fishing nets (0.15 m^2) were placed as artificial shelters in each tank to reduce *M. nipponense* cannibalization. The prawns were reared under natural photoperiod of 14 to 12 h light and 10 to 12 h dark. The prawns were observed every day for mortality and the number of dead prawns were collected and recorded daily at 16:00 h. One-third of culture water in each tank was replaced daily with fresh de-chlorinated water to maintain water quality. The water temperature was between 26 °C and 31 °C; dissolved oxygen was maintained above 6.5 mg/L; and ammonia nitrogen and nitrate contents maintained below 0.01 mg/L.

2.3. Proximate composition analysis of the diets

Three replicates of each of the formulated diets were analyzed for proximate composition according to standard methods [32]. The dry matter was measured by drying the feed at 105 °C to constant weight. The crude lipid was extracted and quantified by using the Soxhlet extraction system. Ash was measured by incineration in a muffle furnace at 550 °C for 6 h. Crude protein content was determined by using the elementar rapid N exceed (Elementar Analysensysteme GmbH., Germany).

2.4. Growth performance and survival of prawns under normoxia

At the end of the feeding trial, all survived prawns were counted to determine survival rate and weight was measured for calculating weight gain, and specific growth rate, as follows:

Survival rate (%) = 100 × final number of prawns / initial number of prawns

Weight gain (%) = 100 × (final weight - initial weight) / initial weight

Specific growth rate (% /d) = 100 × (Ln final weight – Ln initial weight) / time (days)

2.5. Acute hypoxia stress and reoxygenation of prawns

After growth performance and survival rate measurements, 90 prawns were randomly sampled from each treatment for acute hypoxia stress. The prawns were randomly assigned to 15 tanks (75 × 55 × 60 cm in length, width and height, respectively) in three replicates and acclimated for two days. Each tank was filled with 150 L of de-chlorinated water. Each tank was provided with two air stones, one for supplying oxygen and another for nitrogen. Each air stone has a valve that can adjust the flow rate. Each tank was covered with plastic wraps in order to maintain dissolved oxygen into the water. To induce hypoxia, the oxygen air stone valve was closed, and the nitrogen air stone valve was opened to fill nitrogen into the water. The dissolved oxygen was measured by using an oxygen meter YSI-550A (Xylem Inc., USA). During hypoxia, the dissolved oxygen in the water was kept at 2 mg/L (measured as 2.06 ± 0.11 mg/L). The amount of dissolved oxygen was selected based on a previous study on hypoxia stress in crustaceans [33]. The amount of dissolved oxygen in the water was continuously supplied by using two air stones. The prawns were exposed to hypoxia exposure for 12 h.

During reoxygenation, the plastic wraps were removed, the nitrogen air stone valve was closed, and oxygen air stone valve was opened to supply dissolved oxygen in the water to a level of 8.30 ± 0.07 mg/L. The prawns were subjected to reoxygenation for 12 h.

2.6. Determination of carbohydrate metabolism enzymes activities of prawns under normoxia

A sample of 10 prawns were randomly collected from each tank after the feeding trial under normoxia condition to obtain hepatopancreas tissue for analysis of carbohydrate metabolism enzymes activities, antioxidant capacity and mRNA expression of genes. Prawns were sacrificed by tweezers and dissected to obtain the hepatopancreas. The hepatopancreas was obtained from the cephalothorax and then stored in labeled Eppendorf tubes at −80 °C for later analysis. On the days of analyses, crude extracts from the hepatopancreas were obtained by homogenizing the frozen tissue with ice-cold 0.86% (w/v) NaCl solution. The crude extracts were centrifuged for 10 min (Eppendorf Centrifuge 5810 R, speed: 3000 rpm/min) to get the supernatant for carbohydrate metabolism enzymes activities analysis. Part of the hepatopancreas supernatant obtained was stored at −80 °C for later antioxidant enzymes activities analysis.

The succinate dehydrogenase (SDH, EC 1.3.5.4) and phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) in the hepatopancreas were determined by using specific commercial kits purchased from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (Nanjing, China) according to manufacturer's instructions. The glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) in the hepatopancreas was determined by using a specific commercial kits purchased from the Suzhou

Keming Biotechnology Co., Ltd. (Suzhou, China) based on protocols given by the manufacturer. Absorbances during analyzes were measured by using a full-wavelength microplate reader (Thermo Scientific™ Multiskan™ GO 1510).

2.7. Evaluation of antioxidant enzymes activities under normoxia-hypoxia-reoxygenation conditions

Part of the hepatopancreas supernatant obtained above was used for evaluation of antioxidant enzymes activities analysis. Also, during hypoxia exposure, 10 prawns were randomly sampled from each tank after 12 h. Likewise, 10 prawns were also randomly sampled during reoxygenation after 12 h. The prawns were sacrificed as described above and dissected to obtain the hepatopancreas supernatant as described above for evaluation of antioxidant capacity. The hepatopancreas supernatant obtained during normoxia, hypoxia and reoxygeneation was used for determination of superoxide dismutase (SOD, EC 1.15.1.1) and total antioxidant capacity (T-AOC) activities and the malonaldehyde (MDA) content in the hepatopancreas by using specific commercially available kits (Nanjing Jiancheng Bioengineering Institute Co., Ltd., China) following manufacturer's instructions. Absorbances during analyses were measured by using a full-wavelength microplate reader (Thermo Scientific™ Multiskan™ GO 1510).

2.8. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR) in the hepatopancreas under normoxia-hypoxia-reoxygenation conditions

Total RNA was extracted from hepatopancreas from three samples per treatment by using RNAiso Plus (Takara Bio Inc., Japan) according to the manufacturer's protocol. The total RNA concentration and quality were estimated using the Thermo NanoDrop 2000 (Thermo Fisher Scientific (China) Ltd., China) and agarose gel electrophoresis. If the ratio of A260/A280 is within the range specified by the kit (1.7–2.1), first use Recombinant DNase I (RNase-free) (Takara Bio Inc., Japan) to remove genomic DNA, and then use Prime Script™ RT-PCR kit (Takara Bio Inc., Japan) to synthesize cDNA. The qRT-PCR analysis was conducted by using a FastSYBR Mixture (Cwbio Co., Ltd., China) in the CFX96™ Touch Real-Time PCR System (Bio-Rad Laboratories, Inc., USA). PCR conditions were as follows: 95 °C for 10min, and followed 40 cycles of 95 °C for 10s, 58 °C for 30s, and 72 °C for 32s. Samples were run in triplicate and normalized to the control gene the 18S ribosomal RNA (*18S rRNA*) [34]. After amplification, the transcripts of these genes were calculated by using the $2^{-\triangle\triangle Ct}$ method [35]. Primers were designed based on the available cDNA sequences (Table 2) of the oriental river prawn from Genbank and then synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

The AMP-activated protein kinase-alpha (*AMPK-α*), -beta (*AMPK-β*), -gamma (*AMPK-γ*), pyruvate dehydrogenase-E1-α subunit (*PDH-E1-α*) and phosphofructokinase (*PFK*) were detected to determine the effect of thiamine on the mRNA expressions of genes related to energy metabolism under normoxia, hypoxia and reoxygenation conditions. To determine the effect of thiamine on hypoxia-inducible factor and thiamine transporter under normoxia, hypoxia and reoxygenation conditions, *HIF-1α*, -beta (*HIF-1β*) and *SLC19A2* were detected.

Table 2
Primers used in this study.

Primer	Sequence (5'→3')	GenBank	Product
(Mn)18S rRNA-S	TACTGCTGAGCCGAAGAT	EU118285.1	159
(Mn)18S rRNA-A	CCACGGACTATTACTACCTAC		
(Mn)AMPK-α-S	ACAGGGACAAAGGTAGCG	MG792549.1	116
(Mn)AMPK-α-A	ATGTGTGGATGGCGAAAC		
(Mn)AMPK-β-S	TCGTCTCGTAGGGTGTAGG	MG792548.1	191
(Mn)AMPK-β-A	TAGGCTGATGGTAGGTGGA		
(Mn)AMPK-γ-S	CGCTTCTCCCTGTCCCT	MG792547.1	124
(Mn)AMPK-γ-A	CCGCAGCCTGGTAAACT		
(Mn)HIF-1α-S	CGGTAGTTGTAGTCCCTTATC	KP050352.1	169
(Mn)HIF-1α-A	AGTGCTTACTCGACGGTTG		
(Mn)HIF-1β-S	TACCGCCACAACGCAACT	KP050353.1	124
(Mn)HIF-1β-A	ATCATCCGCCGACTCCAT		
(Mn)PFK-S	GCACAAAGGAAAGGGCATCG	KT932420.1	231
(Mn)PFK-A	CACCTGGCAGAACCGCTAA		
(Mn)SLC19A2-S	TTGAAGGGATGGGCTAAGT	ON693254	189
(Mn)SLC19A2-A	AAGGATGGCTGAGCGAGTG		
(Mn)PDH-E1-α-S	TGTGGTTCAGGCAAAGGA	ON693252	99
(Mn)PDH-E1-α-A	ATCACGGGTTCGGTAGGA		

2.9. Statistical analyses

All data obtained were tested for normality and homogeneity of variances by using Shapiro Wilk and Levene's tests, respectively. The data obtained were analyzed by one-way analysis of variance (ANOVA). Tukey's test was used for multiple comparisons when ANOVA detected significant difference on measured parameters among the dietary treatments. Two-way ANOVA was used to determine any significant interaction between the concentration of dietary thiamine levels and exposure methods (normoxia, hypoxia and reoxygenation). Differences were considered significant at $P < 0.05$. The results are expressed as mean ± standard deviation (S.D). Linear, quadratic and cubic orthogonal polynomial contrasts were performed for data obtained on MDA content and T-AOC activities, in order to understand the appropriate equation for estimating the optimum thiamine in *M. nipponense* juvenile (Table S1) [36]. The optimum dietary thiamine required in juvenile *M. nipponense* was determined by cubic regression analysis based on MDA content and T-AOC activity. All analyses were conducted by using SPSS 25.0 statistical software (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Growth performance and survival of prawns

The dietary thiamine levels used in the present study did not significantly affect the weight gain, specific growth rate, and survival rate in juvenile *M. nipponense* ($P > 0.05$; Table 3).

3.2. Carbohydrate metabolism enzymes activities during normoxia

The activities of SDH (Fig. 1A), G6PDH (Fig. 1B) and PEPCK (Fig. 1C) decreased significantly as dietary thiamine levels from 5.69 mg/kg to 133.67 mg/kg or 268.33 mg/kg, then increased significantly with further increasing thiamine levels. The prawns fed the 133.67 mg/kg thiamine diet had significantly lower SDH activity than those fed the 5.69, 268.33 and 532.00 mg/kg thiamine diets ($P < 0.05$). The G6PDH activity of prawns fed 268.33 mg/kg thiamine was significantly lower than that of prawns fed the control diet (5.69 mg/kg thiamine) ($P < 0.05$). The PEPCK activity of prawns fed the 133.67 mg/kg thiamine diet decreased significantly compared with those fed the 5.69, 70.70 and

Table 3
Growth performance of juvenile *Macrobrachium nipponense* fed with experimental diets containing different concentrations of thiamine for eight weeks.

Thiamine (mg/kg)	Initial weight (g)	Final weight (g)	Weight gain (%)	Specific growth rate (%/d)	Survival rate (%)
5.69	0.123 ± 0.01	0.54 ± 0.04	336.82 ± 29.77	2.63 ± 0.12	71.67 ± 1.67
70.70	0.123 ± 0.01	0.59 ± 0.03	382.25 ± 24.39	2.81 ± 0.09	76.67 ± 3.34
133.67	0.123 ± 0.01	0.59 ± 0.03	377.31 ± 21.22	2.79 ± 0.08	78.89 ± 2.55
268.33	0.123 ± 0.01	0.52 ± 0.07	325.32 ± 60.78	2.57 ± 0.25	78.34 ± 5.77
532.00	0.123 ± 0.01	0.54 ± 0.08	341.66 ± 66.05	2.87 ± 0.13	72.78 ± 9.18

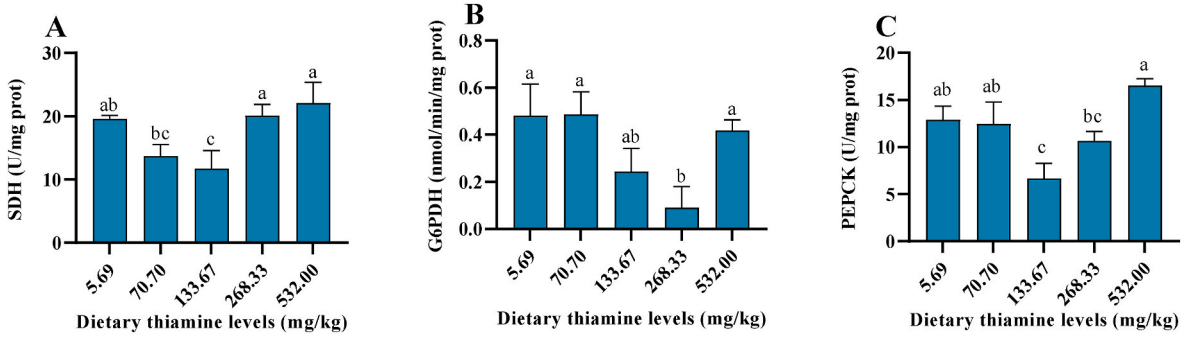


Fig. 1. Carbohydrate metabolism in the hepatopancreas of juvenile *Macrobrachium nipponense* fed with experimental diets containing five levels of thiamine diets for eight weeks under normoxia. A: SDH, succinate dehydrogenase; B: G6PDH, glucose-6-phosphate dehydrogenase; C: PEPCK, phosphoenolpyruvate carboxykinase. Data are mean ± SD. Different letters above bars indicate significant difference ($P < 0.05$).

532.00 mg/kg thiamine diets ($P < 0.05$). There were no statistical differences for the SOD, G6PDH and PEPCK activities between prawns fed the 532.00 mg/kg thiamine and prawns fed the control diet ($P > 0.05$).

3.3. Antioxidant capacity under normoxia-hypoxia-reoxygenation conditions

Dietary thiamine significantly affected the antioxidant capacity of *M. nipponense* under normoxia, hypoxia and reoxygenation conditions (Fig. 2). Under normoxia, the T-AOC activity initially increased significantly with the increased dietary thiamine level, peaking at 133.67 mg/kg diet, and then decreased significantly ($P < 0.05$) (Fig. 2A). Prawns fed the 70.70 to 532.00 mg/kg thiamine diets significantly reduced the MDA content than those fed the 5.69 mg/kg thiamine diet ($P < 0.05$; Fig. 2D). However, the prawns fed the 532.00 mg/kg thiamine diet significantly decreased the SOD activity compared with those fed other thiamine levels ($P < 0.05$) (Fig. 2E). According to cubic regression analysis, the optimum thiamine supplementation was 161.20 mg/kg for minimum MDA content (Fig. 2B) and 143.17 mg/kg for maximum T-AOC activity under normoxia (Fig. 2 C).

Under 12 h hypoxia, prawns fed the 268.33 and 532.00 mg/kg thiamine diets significantly increased the MDA content and the SOD activity than those fed the 5.69, 70.70 and 133.67 mg/kg thiamine diets ($P < 0.05$).

After 12 h reoxygenation, the prawns fed the low (control, 5.69 mg/kg) or high (532.00 mg/kg) thiamine level diet had significantly higher MDA content than those fed the 133.67 and 268.33 mg/kg thiamine diets ($P < 0.05$). Compared with the control prawns, a significantly higher SOD activity of prawns fed the 70.70 mg/kg thiamine diet or a significantly lower SOD activity of prawns fed the 268.33 mg/kg thiamine diet was observed after 12 h of reoxygenation ($P < 0.05$). The thiamine concentrations and exposure methods interacted significantly

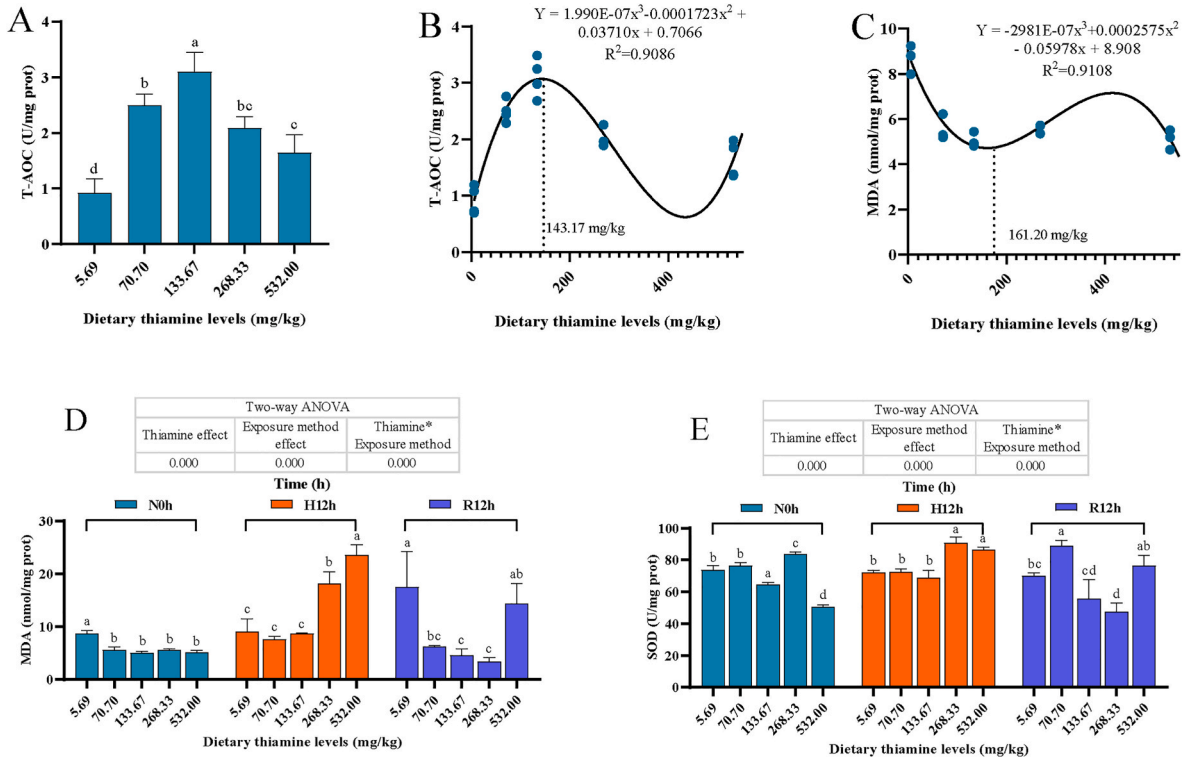


Fig. 2. The antioxidant status in the hepatopancreas of *Macrobrachium nipponense* fed diets supplemented with five thiamine concentration under normoxia-hypoxia-reoxygenation treatments. A: total antioxidant capacity (T-AOC), B: Optimum amount of thiamine based on T-AOC activity; C: Optimum amount of thiamine based on malondialdehyde (MDA) content; D: MDA content, E: superoxide dismutase (SOD) activity. N0h: 0 h of normoxia; H12h: 12 h of hypoxia; R12h: 12 h of reoxygenation. Data are mean ± SD. Different letters above bars indicate significant difference ($P < 0.05$).

to affect MDA content and SOD activity ($P < 0.01$).

3.4. The mRNA expressions of genes related to energy metabolism in the hepatopancreas under normoxia-hypoxia-reoxygenation conditions

Dietary thiamine significantly affected the mRNA expressions of energy metabolism related genes in the hepatopancreas of *M. nipponense* during normoxia, hypoxia and reoxygenation conditions ($P < 0.05$; Fig. 3).

Under normoxia, prawns fed the 133.67, 268.33 and 532.00 mg/kg thiamine diets significantly downregulated mRNA expression of *AMPK-α* gene (Fig. 3A) compared with the control diet ($P < 0.05$). No significant difference was obtained for the mRNA expression of *AMPK-β* gene among the prawns fed the 5.69, 133.67, 268.33 and 532.00 mg/kg thiamine diets (Fig. 3B). Compared with the control group, the prawns fed the 70.70 and 133.67 mg/kg thiamine diets significantly downregulated the mRNA expression of *AMPK-γ* gene ($P < 0.05$) (Fig. 3C). The *PFK* mRNA expression in prawns fed the 133.67 and 268.33 mg/kg thiamine diets increased significantly compared with that of prawns fed the control diet ($P < 0.05$) (Fig. 4A). *PDH-E1-α* expression in prawns fed the 70.70 and 133.67 mg/kg thiamine diets was significantly lower than that of prawns fed the 5.69, 268.33 and 532.00 mg/kg thiamine ($P < 0.05$) (Fig. 4B).

Under 12 h hypoxia, prawns fed the 70.70 and 133.67 mg/kg thiamine diets significantly downregulated the mRNA expression of *AMPK-α* than those fed the control diet ($P < 0.05$). Prawns fed the 70.70, 133.67 and 532.00 mg/kg thiamine diets also significantly downregulated the mRNA expression of *AMPK-β* gene after 12 h hypoxia than those fed the 5.69 and 268.33 mg/kg thiamine diets ($P < 0.05$). However, *AMPK-γ* expression in prawns fed the 268.33 and 532.00 mg/kg thiamine diets increased significantly after 12 h of hypoxia compared with that of prawns fed the control diet ($P < 0.05$). *PFK* and *PEH-E1-α* expressions in prawns fed the 532.00 mg/kg thiamine diet also upregulated significantly after 12 h hypoxia ($P < 0.05$).

After 12 h reoxygenation, prawns fed the control diet had the lower mRNA expression of *AMPK-α* than all other diets ($P < 0.05$). Likewise, prawns fed the control diet also significantly downregulated the mRNA expression of *AMPK-β* compared with those fed 133.67, 268.33 and 532.00 mg/kg thiamine diets ($P < 0.05$). Prawns fed the 70.70 mg/kg thiamine diet significantly upregulated the mRNA expression of *AMPK-γ* after 12 h of reoxygenation than all other diets ($P < 0.05$). *PFK* mRNA expression in prawns fed the 268.33 mg/kg thiamine diets increased significantly compared with that of prawns fed the control diet after 12 h of reoxygenation ($P < 0.05$). Similarly, *PDH-E1-α* mRNA expression in prawns fed the 70.70, 133.67 and 268.33 mg/kg thiamine diets was also upregulated after 12 h of reoxygenation compared with those fed the 5.69 and 532.00 mg/kg thiamine diets ($P < 0.05$). The thiamine concentrations and exposure methods interacted significantly to affect the *AMPK-α, AMPK-β, AMPK-γ, PFK* and *PDH-E1-α* genes ($P < 0.01$).

3.5. The mRNA expression of HIF-1 and SLC19A2 in the hepatopancreas under normoxia-hypoxia-reoxygenation conditions

Under normoxia, *M. nipponense* fed the control diet upregulated mRNA expression of *HIF-1α* than those the fed other diets ($P < 0.05$) (Fig. 5A). The expression of *HIF-1β* decreased significantly as dietary thiamine levels increased from 5.69 to 133.67 mg/kg, and then increased significantly with further increasing thiamine levels ($P < 0.05$) (Fig. 5B). Feeding *M. nipponense* with all the experimental diets did not significantly affect the mRNA expression of *SLC19A2* gene (Fig. 5C; $P > 0.05$).

Under hypoxia, prawns fed the control diet significantly upregulated the mRNA expressions of *HIF-1α, HIF-1β* and *SLC19A2* than those fed other diets ($P < 0.05$). After 12 h reoxygenation, feeding the dietary thiamine did not significantly affect the mRNA expression of *HIF-1α* gene ($P > 0.05$). However, *M. nipponense* fed the 268.33 mg/kg thiamine diet downregulated the mRNA expression of *HIF-1β* after 12 h of reoxygenation than those fed the 5.69, 70.70 and 133.67 mg/kg thiamine diets ($P < 0.05$). The prawns fed the 5.69 and 268.33 mg/kg thiamine diet downregulated the mRNA expression of *SLC19A2* gene after 12 h of reoxygenation than those fed the 133.67 and 532.00 mg/kg thiamine diet ($P < 0.05$). The thiamine concentrations and exposure methods interacted significantly to affect the *HIF-1α, HIF-1β* and *SLC19A2* genes ($P < 0.01$).

4. Discussion

In the present study, we found that dietary thiamine did not affect weight gain and survival rate of juvenile *M. nipponense*, similar to the results of *P. japonicus*. [37], but different from the results reported previously on this species by Sun et al. [38]. The difference may be due to the duration of growth trial. Our experimental period was for eight weeks, while that of Sun et al. lasted for 10 weeks [38]. Similarly, a previous study found that before 12 weeks, there was no significant difference in the growth of *Scophthalmus maximus* between the thiamine-supplemented and non-thiamine-supplemented group, but after 12 weeks, the addition of thiamine to the feed significantly promoted the growth of *S. maximus* [39]. Furthermore, poor appetite appeared in *S. maximus* fed a thiamin-deficient diet after 10 weeks [39], and similar phenomenon was also observed in the study on mice [40]. However, in our study, poor appetite of prawns induced by thiamin-deficient did not appear during the whole period of trial, which may be one of reasons for no significant difference of the final growth performance of prawns. Moreover, three types of thiamine (thiamine nitrate, thiamine hydrochloride and benfotiamine) have been studied in many studies presently. It has been suggested that benfotiamine, as a lipid-soluble thiamine derivative has higher bioavailability than thiamine hydrochloride and thiamine nitrate [41]. However, the safety of benfotiamine requires further studies to explore [41]. Furthermore, thiamine nitrate has a higher solubility compared to thiamine hydrochloride [42]. Therefore, in our study, thiamine nitrate was added into the diets of *M. nipponense*. However, the study by Sun et al. did not introduce the thiamine type in their study [38], which also probably affected the comparison results of growth performance of *M. nipponense*.

The antioxidant system is important to maintain the homeostasis of ROS produced in organisms [43]. MDA is a product of lipid peroxidation, and its level is proportional to body oxidative damage [44]. SOD plays a crucial role in protecting cells from oxidative damage by scavenging superoxide anion free radicals. T-AOC is an overall indicator of the antioxidant status of an individual, representing the level of enzyme and non-enzyme original antioxidant in the body [45]. It has been shown that thiamine deficiency causes metabolic abnormalities and disruption of oxidative metabolism, resulting in excessive ROS production in mitochondria [46]. In our study, we found that the MDA level in prawns fed the 5.69 mg/kg thiamine diet (the control diet) was significantly higher than the remaining diets, while the T-AOC activity in control diet was significantly lower than other groups, which suggested that thiamine deficiency caused oxidative stress to *M. nipponense*. Similar results were also observed on *Ctenopharyngodon idella* [47] and *Trachinotus ovatus* [48]. It has been demonstrated that thiamine has direct [20] and indirect [46] antioxidant effects. In this study, we also found that 143.17 to 161.20 mg/kg thiamine supplementation improved T-AOC under normoxia without inducing any oxidative stress to *M. nipponense*, corresponding to the lower MDA content based on cubic regression analysis. Interestingly, we found that when prawns were fed excess thiamine (532.00 mg/kg), the SOD and T-AOC activities were reduced significantly. It seems that excess addition of thiamine may have some adverse effects on the antioxidant system, which need further research.

Thiamine has been reported to reduce ROS production under stressful conditions [49]. However, prawns fed the 268.33 and 532.00 mg/kg thiamine diets enhanced SOD activity after 12 h of hypoxia, but

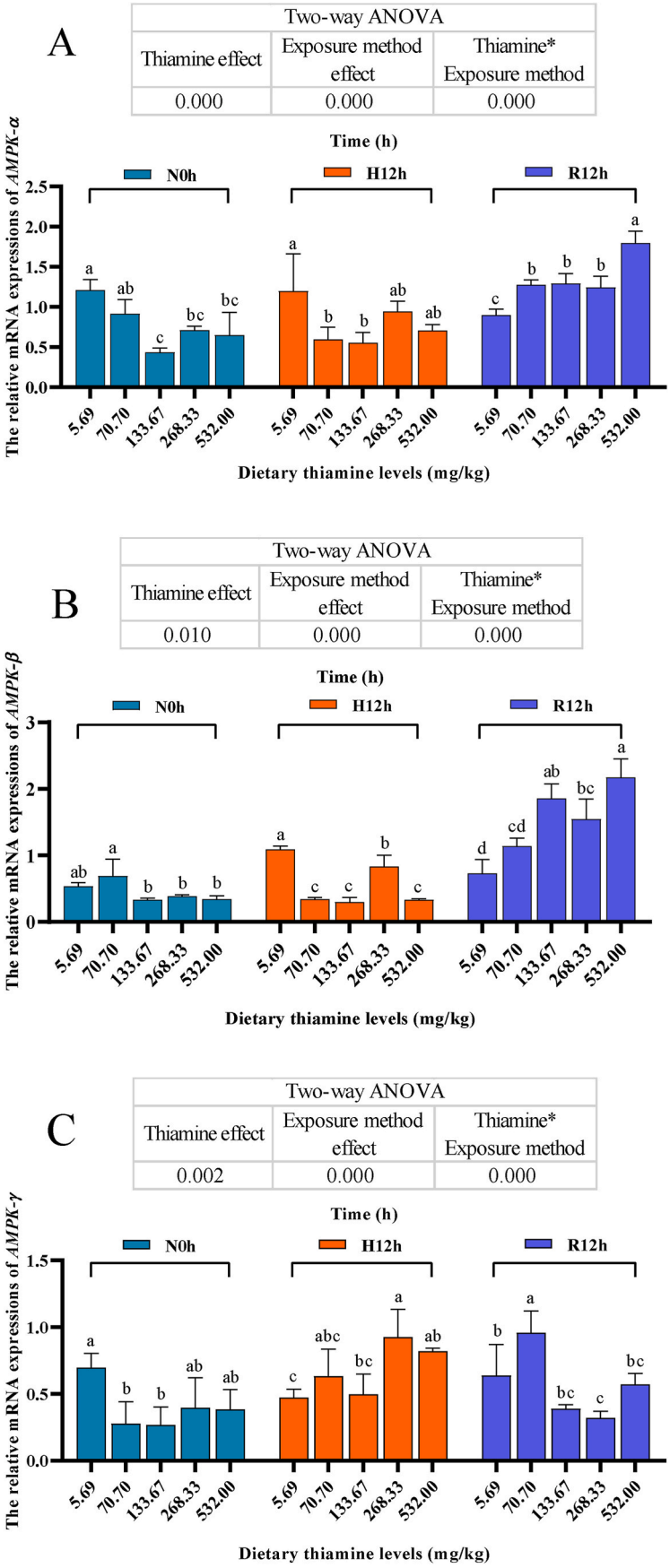
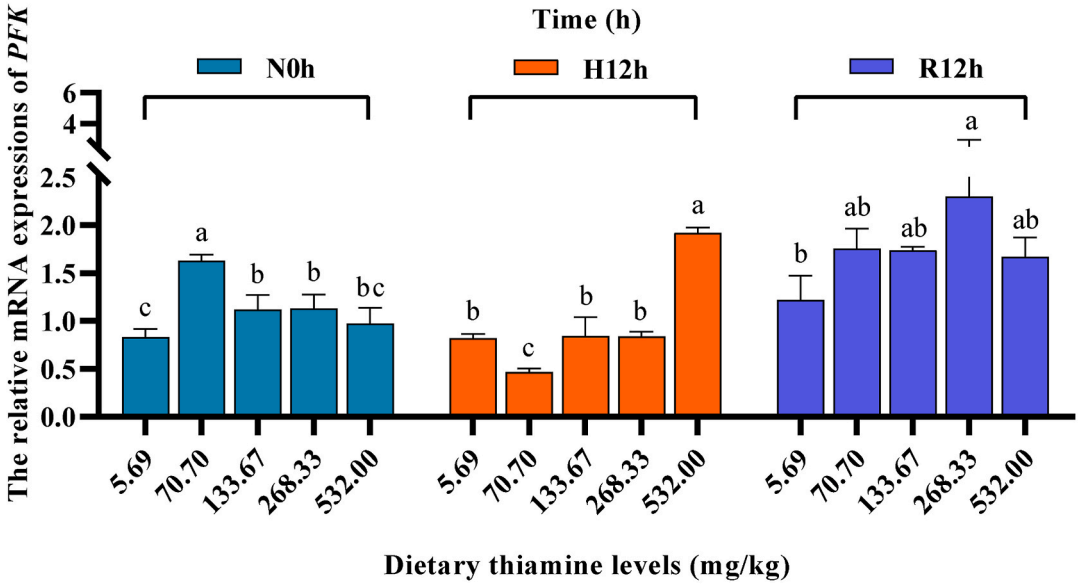


Fig. 3. Effects of dietary thiamine on AMP-activated protein kinase (*AMPK*) expression in the hepatopancreas of *Macrobrachium nipponense* under normoxia-hypoxia-reoxygenation conditions. A: AMP-activated protein kinase-alpha (*AMPK-α*), B: AMP-activated protein kinase-beta (*AMPK-β*), C: AMP-activated protein kinase-gamma (*AMPK-γ*). N0h: 0 h of normoxia; H12h: 12 h of hypoxia; and R12h: 12 h of reoxygenation. Data are mean ± SD. Different letters above bars indicate significant difference ($P < 0.05$).

A

Two-way ANOVA		
Thiamine effect	Exposure method effect	Thiamine* Exposure method
0.000	0.000	0.000



B

Two-way ANOVA		
Thiamine effect	Exposure method effect	Thiamine* Exposure method
0.000	0.000	0.000

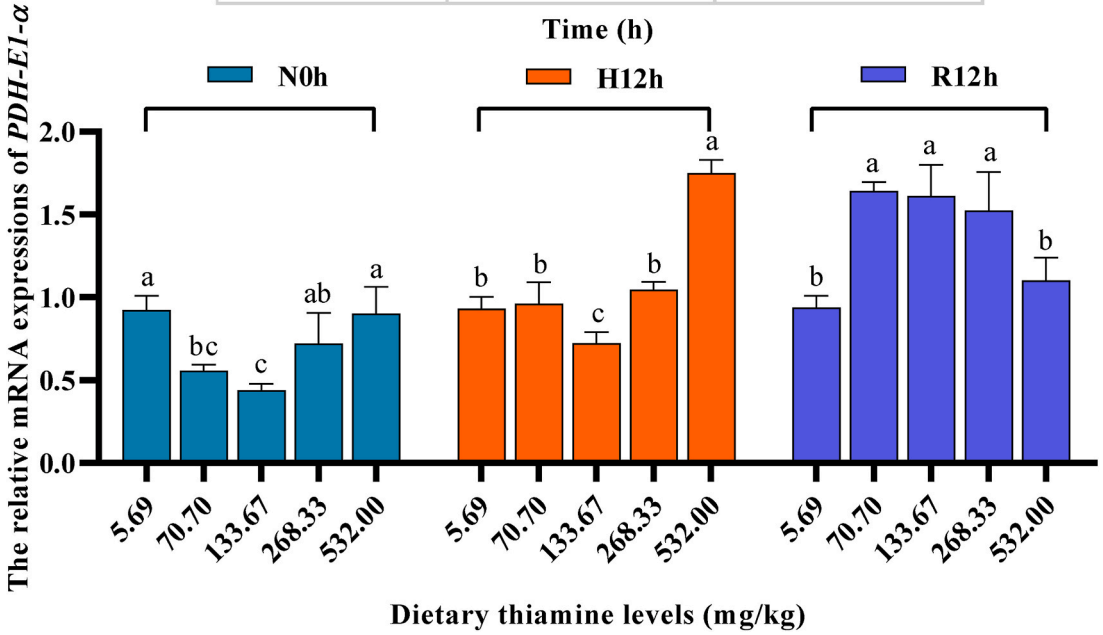


Fig. 4. Effects of dietary thiamine on carbohydrate metabolism in the hepatopancreas of *Macrobrachium nipponense* under normoxia-hypoxia-reoxygenation conditions. A: phosphofructokinase (*PFK*), B: pyruvate dehydrogenase-E1-α subunit (*PDH-E1-α*). N0h: 0 h of normoxia; H12h: 12 h of hypoxia; and R12h: 12 h of reoxygenation. Data are mean ± SD. Different letters above bar indicate significant difference ($P < 0.05$).

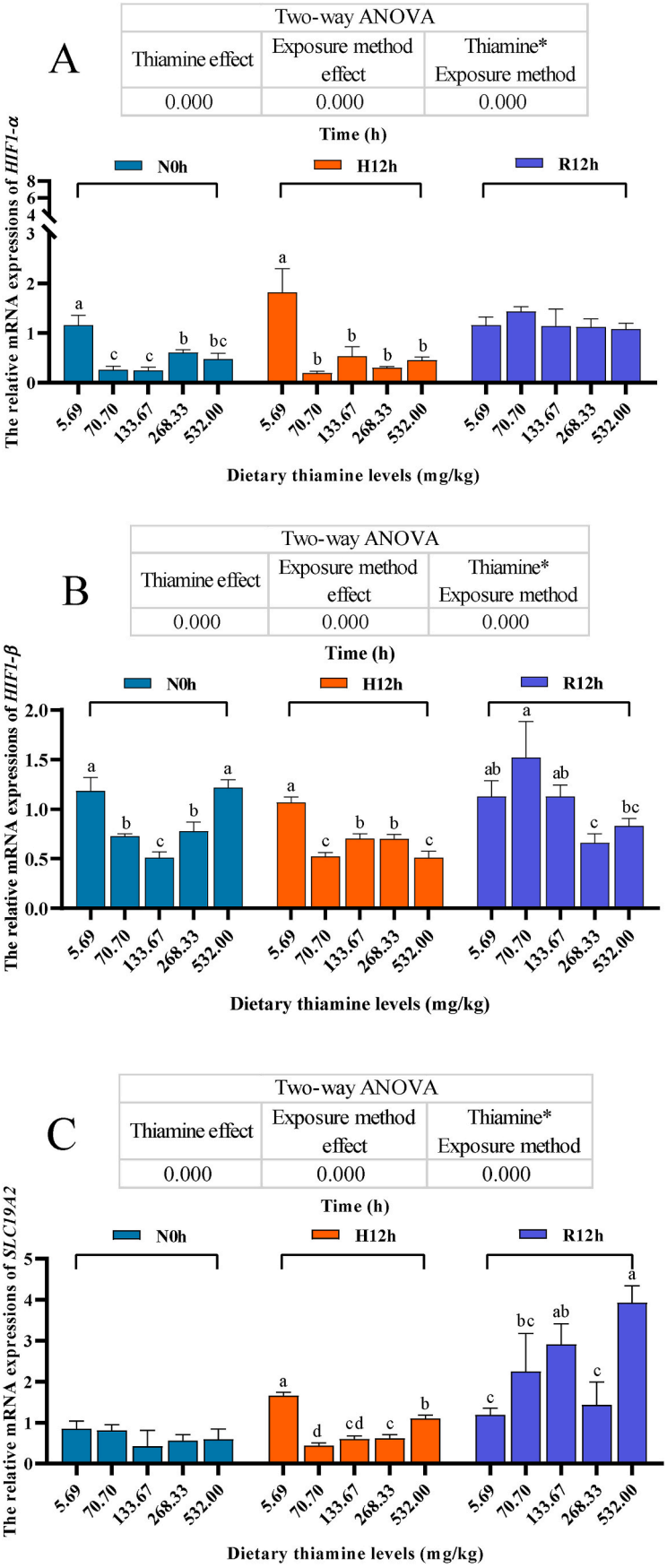


Fig. 5. Effects of dietary thiamine on hypoxia-inducible factor-1 and solute carrier family 19 member 2 in the hepatopancreas of *Macrobrachium nipponense* under normoxia-hypoxia-reoxygenation conditions. A: hypoxia-inducible factor-1 alpha (*HIF-1α*), B: hypoxia-inducible factor-1 beta (*HIF-1β*), C: solute carrier family 19 member 2 (*SLC19A2*). N0h: 0 h of normoxia; H12h: 12 h of hypoxia; R12h: 12 h of reoxygenation. Data are mean ± SD. Different letters above bars indicate significant difference ($P < 0.05$).

had higher MDA content than 70.70 and 133.67 mg/kg thiamine diets. These results suggest that thiamine supplementation probably induced oxidative stress at higher levels. It is reported that, under hypoxia conditions, an increase in acetyl-CoA in TCA cycle usually causes oxidative stress [50]. As mentioned before, PDH catalyzes the conversion of pyruvate into acetyl-CoA [11]. Therefore, the possibility of hypoxia condition causing oxidative stress in *M. nipponense* can not be excluded due to the increased acetyl-CoA, which can be seen as evidenced by the increased mRNA expression of *PDH-E1-α* of prawns fed high level of thiamine under 12 h of hypoxia. Accordingly, excessive thiamine in our study probably exacerbated oxidative stress, as demonstrated by the highest MDA level of prawns fed the 532.00 mg/kg thiamine diet after 12 h of hypoxia. We hypothesize that, the increased SOD activity of prawns fed the high level of thiamine at this time may be the response of cells to oxidative stress. Therefore, the excess of thiamine supplementation was not conducive to the recovery of *M. nipponense* from hypoxic stress.

Hypoxia induces a shift in metabolism towards a reliance on glycolysis to meet bioenergetics requirement in cells [15]. Increased glycolysis is an important compensatory response to ensure energy supply during stress [51]. Thiamine affected carbohydrate metabolism under normal and hypoxia status in our study. PFK is a rate-limiting enzyme during glycolysis [38]. During normoxia, the *PFK* expression increased remarkably for prawns fed the 70.70 mg/kg thiamine dietary level, suggesting enhanced glycolysis rate at this supplementation level. However, supplementing 70.70 and 133.67 mg/kg thiamine diets decreased *PDH-E1-α* expression under normoxia, which suggested reduced entry of pyruvate into the TCA cycle. Accordingly, inhibiting PDH reduced mitochondrial damage during hypoxia stress and ischemia-reperfusion injury [8]. Interestingly, *M. nipponense* supplemented with 268.33 mg/kg thiamine diet simultaneously increased *PDH-E1-α* and *PFK* genes after 12 h of reoxygenation without causing oxidative stress. The increased *PFK* indicated that thiamine supplementation alleviated energy deficiency by enhancing glycolysis [52], while the increased *PDH-E1-α* suggested that thiamine enhanced the amount of pyruvate entering TCA to exacerbate oxidative stress as mentioned above. Therefore, supplementing 70.70 to 133.67 mg/kg thiamine to the diet of *M. nipponense* resist hypoxia stress by regulating carbohydrate metabolism.

PEPCK is the rate-limiting enzyme of gluconeogenesis pathway [53]. In the present study, the 133.67 mg/kg thiamine diet inhibited gluconeogenesis in *M. nipponense*, similar to previous results [38]. However, when thiamine supplementation was further increased, gluconeogenesis was enhanced. Thiamine has been reported to promote absorption and utilization of glucose in the body [54]. Therefore, we hypothesize that increased gluconeogenesis occurred in hepatopancreas was in order to maintain glucose stability in *M. nipponense* hemolymph.

The pentose phosphate pathway provides pentose for ribonucleotide synthesis and generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) to participate in the synthesis of DNA, amino acids, fatty acids and other biomolecules [55]. G6PDH is the rate-limiting enzyme for the pentose phosphate pathway [56]. In our study, G6PDH activity was reduced in prawns fed the 268.33 mg/kg thiamine diet, suggesting no need for more NAPDH at this supplementation level. SDH is one of the hubs connecting oxidative phosphorylation and electron transport [57]. The reduced SDH activity in prawns fed the 133.67 mg/kg thiamine diet, might be a response to increase energy production due to lowered pyruvate entry into the TCA cycle. Overall, under normoxia and hypoxia conditions, the optimum thiamine requirement varies in order to maintain *M. nipponense* metabolic homeostasis.

The effects of hypoxia on glycolysis may be coordinated through AMPK and HIF-1 [58]. Adenosine AMPK is one of the key proteins involved in regulating energy metabolism during hypoxia. AMPK is made up of three subunits, which are AMPK-α, a catalytic subunit, AMPK-β, a scaffolding subunit, and AMPK-γ, a regulatory subunit [59]. Hypoxia, ROS and nutrients depletion are known to activate AMPK [60]. Under normoxia condition, thiamine supplementation inhibited the *AMPK-α*, *AMPK-β* and *AMPK-γ* expression in *M. nipponense*, suggesting the prawns was in a suitable internal cell environment. Interestingly, hypoxia condition did not activate *AMPK-α and AMPK-β* expression except *AMPK-γ*. Therefore, we speculate that thiamine can alleviate hypoxia by regulating *AMPKs*.

HIF-1 plays an important role in protecting aquatic animals against hypoxia stress [61]. During hypoxia and reoxygenation conditions, *HIF-1α* stability makes animals survive better under hypoxia stress [62]. There was a corresponding increase in *HIF-1α* stabilization during thiamine deficiency [63]. Our study showed that thiamine supplementation decreased the *HIF-1α* and *HIF-1β* mRNA expression, suggesting thiamine attenuated hypoxia stress in *M. nipponense*. The underlying mechanism of alleviation of hypoxia by thiamine may be closely related to thiamine transport, because HIF-1α can bind and activate SLC19A3 in response to hypoxia stress [15]. In the present study, we found that the mRNA expression of *SLC19A2* was consistent with *HIF-1α* during hypoxia condition (except for the 532.00 mg/kg). The synchrony on the expression of these two genes suggests that thiamine is important in hypoxia regulation. Therefore, appropriate thiamine supplementation alleviates hypoxia stress as indicated by lower levels of *HIF-1α* and *SLC19A2* expression, which is beneficial for the cells.

5. Conclusion

In summary, the optimum thiamine was 161.20 mg/kg and 143.17 mg/kg based on cubic regression analysis of MDA content and T-AOC activity *vs.* dietary thiamine level, respectively. Supplementing 143.17 to 161.20 mg/kg thiamine in the diets for *M. nipponense* improves the antioxidant capacity under normoxia and reduces the oxidative damage under hypoxia stress. The 70.70 mg/kg thiamine diet improves carbohydrate metabolism in *M. nipponense*, while 133.67 mg/kg of thiamine alleviates hypoxia stress by balancing energy production and consumption.

Declaration of interest

None.

CRediT authorship contribution statement

Dongsheng Zhou: Enzyme activities determination and manuscript drafting. **Chengli Wang:** manuscript drafting. **Jinxian Zheng:** Enzyme activities determination. **Jianhua Zhao:** manuscript revision. **Shanshan Wei:** Enzyme activities determination. **Yunfeng Xiong:** Data analysis and feeding trial. **Samwel Mchele Limbu:** Manuscript revision. **Youqin Kong:** Manuscript revision. **Fang Cao:** Manuscript revision. **Zhili Ding:** Experiment design and manuscript revision.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.09.059.

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