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Dietary L-carnitine improves glycogen and protein accumulation in Nile tilapia via increasing lipid-sourced energy supply: An isotope-based metabolic tracking

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ABSTRACT

L-carnitine is a functional aquafeed additive for enhancing lipid catabolism by elevating mitochondrial fatty acid β-oxidation and modulating energy metabolism to provide a "protein sparing effect". However, results on the effects of dietary L-carnitine on nutrient metabolism in fish are still conflicting. We explored comprehensively the effects of dietary L-carnitine on energy metabolism in Nile tilapia. We fed Nile tilapia for eight weeks with diets supplemented with L-carnitine or not. We conducted metabolic tracking tests by intraperitoneally injecting individual fish with ^{14}C-labeled palmitic acid (PA), glucose (Glu) and an amino acid mixture (AAs). After the feeding trial, insignificant growth-promoting effect of L-carnitine was obtained in treated fish. However, L-carnitine significantly reduced the lipid content in whole body and muscle accompanied by increasing the free carnitine concentration and fatty acid β-oxidation efficiency. Moreover, L-carnitine elevated concentrations of serum glucose, pyruvate and lactate, and increased glycogen and protein deposition in muscle. These results suggest that ingested glucose and protein prefer to be reserved in carnitine-fed fish with sufficient fatty acids oxidation for energy. Nevertheless, after a ^{14}C-labeled single nutrient injection, carnitine-fed fish showed a higher oxidation rate of [1-^{14}C]-PA, D-[1-^{14}C]-Glu and L-[^{14}C (U)]-AAs. Our study indicates that, the effects of L-carnitine on nutrient metabolism are correlated with the abundance of individual macronutrients such that an inadequate lipid supply would cause dietary L-carnitine supplementation to elevate higher breakdown of glucose and protein for energy generation. The present study provides new insights on the regulation mechanism of L-carnitine on nutrient metabolism in fish.

1. Introduction

In classic fish nutrition theory, the "protein sparing effect" is referred to as any manipulation intended to minimize protein catabolism including addition of carbohydrates and fats to a low-protein diet (Li et al., 2012). In the current aquafeed industry, protein is the most expensive macronutrient in feeds formulation. The increasing cost and limited supplies of traditional protein sources, such as fishmeal, have increased the trend of using high carbohydrate or fat diets in aquafeeds production to maximize a "protein sparing effect" (Francis and Turchini, 2017). However, the use of high-fat diets (HFD) has caused some metabolic diseases, including excess lipid accumulation in the liver and abdominal adipose tissues (Du et al., 2010; Yan et al., 2015; Cao et al., 2019), which impair fish immune functions and resistance to stress (Jin et al., 2013; Dai et al., 2018). Therefore, improved lipid catabolism has been an important research topic in order to ensure an efficient energy supply so as to promote protein deposition and reduce fat accumulation.

L-carnitine (L-β-hydroxy-γ-N, N, N-trimethylaminobutyric acid) is a functional additive, which plays an essential role in the translocation of long-chain fatty acids (LCFAs) from the cytosol into the mitochondrial matrix for subsequent β-oxidation, thus allowing cells to break down fat and obtain energy (Fritz and McEwen, 1959; Porter et al., 2017). In all animals, including fish, L-carnitine can be synthesized naturally from lysine and methionine (Bremer, 1983; Li et al., 2019). Despite the ability of animals to biosynthesize L-carnitine, dietary L-carnitine supplementation is usually necessary, especially when body lipid content exceeds the animal's capacity to synthesize L-carnitine (Harpaz, 2005;

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Zheng et al., 2014). Thus, L-carnitine is regarded as a conditionally essential nutrient for animals.

During the past decades, the effects of dietary L-carnitine on nutrient metabolism have been studied extensively in different farmed fish species. L-carnitine was reported to reduce body lipid content (Keshavanath, 1998; Singh et al., 2008; Desai et al., 2010; Yang et al., 2012; Sharifzadeh et al., 2017), and to increase body and muscle protein content, showing a significant protein sparing effect (Ozório et al., 2003; Harpaz, 2005; Zhang et al., 2009; Li et al., 2019). Moreover, L-carnitine has also been shown to affect glucose metabolism by influencing blood glucose level and tissues glycogen content (Sharifzadeh et al., 2017). Similarly, some studies from our laboratory have indicated that dietary L-carnitine supplementation not only enhanced lipid catabolism by elevating mitochondrial fatty acid β-oxidation, but also lowered glucose utilization and promoted protein synthesis (Li et al., 2017; Lu et al., 2019). We also demonstrated that L-carnitine deficiency in fish, which was induced by a specific carnitine synthesis inhibitor (mildronate, MD), increased hepatic lipid content (Pan et al., 2017; Li et al., 2018) and decreased whole body glycogen content (Li et al., 2018). These results indicate that dietary L-carnitine improves lipid catabolism to reduce body fat and affects carbohydrate and protein metabolism to promote protein deposition. However, the results on the effect of dietary L-carnitine on nutrient metabolism in fish are still conflicting. A few studies have reported that dietary L-carnitine increases lipid content in liver and muscle (Selcuk et al., 2010; Kordi et al., 2012; Zheng et al., 2014). Some researchers reported that dietary L-carnitine had no significant effects on protein content (Li et al., 2010; Erdogan et al., 2015), and even caused a negative effect on protein deposition by reducing protein content (Kordi et al., 2012). The underlying mechanisms on the effect of dietary L-carnitine on nutrient metabolism in fish are still poorly understood. Based on the current existing research data, there are two inconsistent viewpoints in explaining the regulatory effects of L-carnitine in nutrient metabolism in fish, either; 1) L-carnitine only promotes mitochondrial fatty acid β-oxidation for fuels, and has no influence on the metabolism of other nutrients, including protein deposition; or 2) L-carnitine simultaneously stimulates metabolism of several macronutrients, and alters the contents of glycogen and protein. In order to better understand these inconsistent points, scientists and practitioners should follow the exact metabolic turnover of several individual nutrient in fish when diets are supplemented with L-carnitine. However, this knowledge is currently missing, which largely limits our understanding on the role of dietary L-carnitine in regulating nutrient metabolism.

Therefore, in the present study, to thoroughly explore the effects of dietary L-carnitine on energy metabolism in fish, we fed Nile tilapia with diets supplemented with L-carnitine for eight weeks. We then evaluated the growth performance, liver and muscle energy metabolic characteristics, as well as intestinal digestive enzymes activities. Moreover, we conducted a metabolic tracking test of macronutrients by intraperitoneal (i.p.) injection individual fish with ^{14}C-labeled palmitic acid (PA), glucose (Glu) and an amino acid mixture (AAs). The comprehensive effects of dietary L-carnitine on the efficiency of oxidation, retention and conversion of macronutrients in fish were investigated for the first time by using an isotope based metabolic tracking method. This study provides novel insights on the functions of L-carnitine on fish nutrition metabolic regulation.

2. Materials and methods

All experiments were conducted under the Guidelines of the Laboratory Animals Use and Care of China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

Table 1
Formulation and proximate composition of experimental diets.

Ingredient (g/kg)	Control (Con)	L-carnitine (Car)
Casein[1]	320	320
Gelatin[2]	80	80
Soybean oil[3]	70	70
Corn starch[4]	300	300
Vitamin premix[5]	10	10
Mineral premix[6]	15	15
Carboxymethyl cellulose[7]	30	30
Cellulose[8]	157.75	157.50
Choline chloride[9]	5	5
Butylated hydroxytoluene[10]	0.25	0.25
$Ca(H_2PO_4)_2$[11]	12	12
L-carnitine[12]	0	0.25
Proximate composition (dry matter basis, %)		
DM	93.20	92.84
Crude lipid	6.60	6.63
Crude protein	31.68	31.70
Ash	5.29	5.40

[1] Casein: Wan Ling, Changzhou Linghao Biotechnology Co., Ltd., Jiangsu, China.
[2] Gelatin: Sangon Biotech (Shanghai) Co., Ltd., China.
[3] Soybean oil: Arawana Brand, Yihai Kerry Investments Co., Ltd., Hubei, China.
[4] Corn starch: Shijiazhuang Tangtian starch Co., Ltd., Hebei, China.
[5] Vitamin premix (Hangzhou Minsheng Bio-Tech Co., Ltd., Zhejiang, China) (mg/kg): 500,000 mg Vitamin A, 50,000 mg Vitamin D3, 2500 mg Vitamin E, 1000 mg Vitamin K3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 mg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Cholin, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C.
[6] Mineral premix (Hangzhou Minsheng Bio-Tech Co., Ltd., Zhejiang, China) (g/kg): 147.4 g $MgSO_4{\cdot}7H_2O$; 49.8 g NaCl; 10.9 g Fe(II) gluconate; 3.12 g $MnSO_4{\cdot}H_2O$; 4.67 g $ZnSO_4{\cdot}7H_2O$; 0.62 g $CuSO_4{\cdot}5H_2O$; 0.16 g KI; 0.08 g $CoCl_2{\cdot}6H_2O$; 0.06 g NH_4 molybdate; 0.02 g $NaSeO_3$.
[7] Carboxymethyl cellulose and.
[8] Cellulose: Shandong Dongda Commerce Co., Ltd., China.
[9] Choline chloride: Sangon Biotech (Shanghai) Co., Ltd., China.
[10] Butylated hydroxytoluene: Sangon Biotech (Shanghai) Co., Ltd., China.
[11] $Ca(H_2PO_4)_2$: Sangon Biotech (Shanghai) Co., Ltd., China.
[12] L-carnitine: Sigma Chemical Co. (St. Louis, MO).

2.1. Experimental fish and diets

Juvenile Nile tilapia were purchased from the Aquaculture Genetics and Breeding Technology center at Shanghai Ocean University (Shanghai, China). Before the experiment, all fish were acclimated in three 200-L tanks under a 14 h light/10 h dark photoperiod at 28 ± 1 °C for two weeks. During this time, fish were fed with a commercial diet (Tognwei, Chengdu, Sichuan province, China) containing ≥ 35 % protein and ≥ 5% lipid. After acclimation, 180 visually healthy male Nile tilapia with relatively similar initial mean weights were selected and randomly distributed into six 200-L tanks (30 fish per tank) in an indoor recirculating freshwater aquaculture system with biology/mechanical filtration. The tanks were filled with water to a volume of 130 L tank^{-1} and the rate of recirculation was 400 L h^{-1}. All fish were divided into two groups (three replicates per group): control (Con, initial body weight 2.31 ± 0.04 g) and L-carnitine (Car, initial body weight 2.30 ± 0.08 g). A purified basal diet was formulated to contain 6.60 % lipid and 31.68 % protein (Table 1). Casein and gelatin were used as protein sources, while soybean oil was used as the lipid source. The experimental diet for fish fed on L-carnitine was formulated to contain 0.25 g of L-carnitine kg^{-1} of diet as previously used (Huang et al., 2010; Li et al., 2019).

2.2. Fish rearing and sampling

During the 8-week feeding trial, fish were hand-fed twice daily

(8:30 a.m. and 18:00 p.m.) at a feeding rate of 4% body weight every day, and kept under a 14 h light/10 h dark photoperiod. The water temperature, dissolved oxygen, pH and total ammonia nitrogen were maintained at ranges from 26–28 °C, 4.8–6.4 mg L^{-1}, 7.5–7.9, and < 0.02 mg L^{-1}, respectively. All fish were weighed every two weeks and the feeding amount was adjusted accordingly. At the end of the experiment, all fish were fasted overnight, and eight fish from each group were anesthetized with MS-222 (tricaine methanesulfonate, 20 mg L^{-1}) and sacrificed for tissue samples collection. Another six fish from each group were anesthetized and kept at −80 °C for body composition analysis. Blood was collected from the caudal vein by using 2 mL syringes (Klmedical, China) and centrifuged (1500 rpm, 10 min). The resulting serum was immediately frozen at −80 °C for further analysis. Liver and muscle tissues from the eight fish were collected from dissected fish and immediately frozen in liquid nitrogen and stored at −80 °C for later measurements. Weight gain (WG), survival rate, feed conversion ratio (FCR), hepatosomatic index (HSI) and mesenteric fat index (MFI) were calculated using the following formulae:

Weight gain (WG, %) = 100 × (final mean body weight - initial mean body weight) / initial mean body weight

Survival rate (%) = 100 × (Final fish number / Initial fish number)

Feed conversion ratio (FCR) = feed intake / (final body weight - initial body weight)

Hepatosomatic index (HSI, %) = 100 × liver weight / body weight

Mesenteric fat index (MFI, %) = 100 × mesenteric fat weight / body weight

2.3. Fish body composition analysis

The total protein content was determined by the Kjeldahl method using a semi-automatic Kjeldahl System (FOSS, Sweden) after acid digestion (Lu et al., 2019). Total fat content in the carcass and whole fish was determined by the chloroform/methanol (2:1, *v/v*) method (Folch et al., 1957).

2.4. Biochemical parameters measurements

The nonesterified free fatty acids (NEFA) in the serum were measured by ELISA kits (R & D Systems, USA). Hepatic and serum triglyceride (TG), serum glucose, pyruvate, lactate, total AAs, hepatic and muscle glycogen contents as well as the activities of intestinal digestive enzymes, including lipase, amylase and protease were evaluated by using specific commercial kits (Jiancheng Biotech Co., Nanjing province, China) according to the manufacturer's instructions. The free carnitine concentration in liver and muscle samples was detected by using liquid chromatography-tandem mass spectrometry analysis (LC–MS/MS) as described in our previous study (Li et al., 2017). Briefly, eight whole liver and parts of muscle from the experimental fish were collected, weighed and homogenized by using distilled water (1:10, *w/v*).The tissue homogenate was centrifuged at 2000 rpm min^{-1} for 15 min. A 100 μl aliquot of homogenate was added directly into distilled water (sample A). Protein in sample A was removed by precipitating with 200 μl of cold acetonitrile (ACN; 1 μg mL^{-1} carbachol, and internal standard, IS) and centrifuged at 10,000 rpm/min for 20 min. A 1 μl of the supernatant was taken for LC–MS/MS analysis. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode, and the MS/MS analysis was performed in the multiple reaction monitoring (MRM) mode, monitoring the transitions of *m/z* 162.0 → 103.0 for carnitine.

2.5. [1-^{14}C]-palmitic acid oxidation in liver and muscle homogenates

At the end of the feeding trial, 12 liver and muscle samples (about 0.1 *g*) from fish were collected from each group, cut finely in ice-cold 0.25 M sucrose buffer containing 2 mM EGTA and 10 mM Tris − HCl, pH 7.4, washed three times in the same buffer, blotted with absorbent paper and weighted. Then, the liver and muscle tissues were diluted in 1:40 and 1:20, *w/v,* respectively, of chilled sucrose medium and homogenized by using a drill-driven Teflon glass homogenizer (Ningbo Scientz Biotechnology Co., China) with 4–6 strokes. A 1 mL homogenate of each sample was used for immediate measurements of palmitic acid β-oxidation at 28 °C as described previously (Ning et al., 2016). In brief, the prepared homogenate samples were mixed with the basic medium and then incubated together for 10 min at 28 °C. The basic medium contained 13.3 mM HEPES, 30 mM KCl, 5 mM $MgCl_2$, 0.7 mM EGTA, 1 mM NAD, 0.1 mM CoASH, 5 mM ATP, and mitochondrial elements (1.0 mM L-carnitine and 25 μM cytochrome C). The palmitic acid β-oxidation reaction was initiated by adding 0.1 mM palmitic acid (supplemented with 0.04 MBq [1-^{14}C]-palmitic acid, specific activity 2.4 MBq $mmol^{-1}$) bound to 100 u M fatty-acid free bovine serum albumin (BSA). After 30 min of incubation at 28 °C, the reaction was terminated by adding 10 % (*w/v*) perchloric acid, which precipitated proteins. The final reaction media was centrifuged at 1200 rpm for 10 min and then the acid-soluble products (ASP, the short metabolites from fatty acid oxidation) were isolated. The rate of palmitic acid oxidation was calculated from the radioactivity of the ASP. A 200 μL of the supernatants were transferred into scintillation vials containing 2 mL of scintillation fluid (Ultima Gold XR, Packard, USA) for radioactivity measurement by using the Tri-Carb 4910TR Liquid Scintillation Analyzer (PerkinElmer).

2.6. Metabolic tracking test by using i.p injected [1-^{14}C]-palmitic acid, D-[1-^{14}C]-Glucose and L-[^{14}C (U)]-amino acid mixture

After the feeding trial, fish were fasted overnight and 18 fish from each group were anesthetized with MS-222 (20 mg L^{-1}). Six fish received an i.p. injection of DMSO containing [1-^{14}C]-palmitic acid (80 mg kg^{-1} BW and 0.1 MBq per 50 g BW, PerkinElmer) (Ning et al., 2016). Another 12 fish were divided into two groups (six fish per group). The fish were injected with saline containing D-[1-^{14}C]-glucose (500 mg kg^{-1} BW and 0.1 M Bq per 50 g BW, PerkinElmer) (Liu et al., 2018) and another saline containing L-[^{14}C (U)]-AAs mixture (500 mg kg^{-1} BW and 0.1 MBq per 50 g BW, PerkinElmer) (Li et al., 2018), respectively. The ratio of unlabeled AAs mixture was 2.7: 2: 1: and 1: 1.3 for L-Lys: L-Arg: DL-Met: and L-His: L-Val, respectively (Sigma, Germany), as previous described (Li et al., 2018). The injected fish were moved immediately into a closed glass jar containing oxygen-saturated water, which was connected to another glass bottle containing 1 N KOH solution. The details of the experimental process have been previously described (Yoshida and Harper, 1960; Garin et al., 1987). In brief, 1 mL KOH solution absorbed $^{14}CO_2$ released from the oxidation of the ^{14}C-labeled nutrient was collected during 90 min. All fish from each group were collected and each individual fish was ground by using a mincing machine for the ^{14}C tracking assays. Each whole fish ground sample (0.5 g) was digested by using a digestion solvent (30 % H_2O_2/$HClO_4$, 1:2, *v/v*) (1:5, *w/v*) at 60 °C in a water bath for 6 h. The ^{14}C-protein (1 mM NaCl-Tris − HCl/10 % $HClO_4$ extraction method), ^{14}C-lipid (chloroform-methanol extraction method, 2:1, *v/v*) and ^{14}C-glycogen (70 % ethanol precipitation method) were extracted as described previously (Challiss et al., 1983; Chan and Krebs, 1985; Olga et al., 2012). After the extraction, the ^{14}C-labeled macronutrients were dissolved into 0.5 mL of strong lysate (30 % H_2O_2/$HClO_4$, 1:2, *v/v*). The radioactivity of 200 u L KOH solution and nutrient lysate were measured after mixing with 2 mL scintillation fluid (Ultima Gold XR, Packard, USA) by using the Tri-Carb 4910TR Liquid Scintillation Analyzer (PerkinElmer).

2.7. Histological analysis of glycogen in muscle

After sampling, small pieces (~1 cm^3) of muscle were fixed immediately in muscle tissue fixative (Wuhan Servicebio Technology Co. China) for 24–48 h at room temperature. The fixed tissues were trimmed into appropriate sizes and shapes and placed in embedding cassettes. The process of paraffin sectioning was conducted as described before (Ning et al., 2016). Briefly, the tissue was followed by dehydration in a series of increasing ethanol concentrations at 50 % (12 h), 70 % (5 h), 80 % (2 h), 90 % (2 h), 95 % (1 h), and 100 % (30 min over two consecutive times), and then transferred to 100 % xylene for transparency, processed and embedded in paraffin wax. The muscles were sliced transversely into 5 μm sections and stained by using periodic acid-schiff (PAS) mixture, and examined under a light microscope (Nikon Ds-Ri2).

2.8. Statistical analyses

Results are presented as mean ± standard error of the mean (SEM). Data were tested for normality by using the Shapiro-Wilk test and homogeneity of variance using Levene's test. All data were normally distributed and exhibited homogeneity of variance. Therefore, a two-tailed independent *t*-test was performed to evaluate the significant differences of measured variables between the Con and Car groups. A significance level of $p \leq 0.05$ was chosen. All analyses were conducted by using the Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1. The effects of dietary L-carnitine on growth performance, fatty acid β-oxidation and lipid content in tissues of Nile tilapia

During the feeding trial, fish were in good health and no mortality was observed. Fish fed the carnitine supplemented diet exhibited lower HSI ($p = 0.035$) and MFI ($p = 0.005$) compared to the control. However, dietary supplementation did not affect the WG ($p = 0.428$) and FCR ($p = 0.169$) between the two groups (Fig. 1).

Free carnitine contents in liver ($p = 0.007$) and muscle ($p = 0.000$) were significantly higher in fish fed on carnitine supplemented diet than control (Fig. 2A). Moreover, carnitine supplementation increased the β-oxidation efficiency of [1-^{14}C]-palmitic acid in muscle ($p = 0.049$; Fig. 2B) compared to the control, but significant difference was only obtained in the liver ($p = 0.365$; Fig. 2B). Dietary L-carnitine supplementation decreased significantly the levels of serum TG ($p = 0.001$; Fig. 2C) and NEFA ($p = 0.005$; Fig. 2D) in treated fish compared to control. Moreover, carnitine supplementation also reduced lipid accumulation in treated fish liver ($p = 0.001$; Fig. 2E), muscle ($p = 0.035$; Fig. 2F) and whole body ($p = 0.040$; Fig. 2G) compared to control. These findings suggest that dietary L-carnitine supplementation increases carnitine contents in liver and muscle, and promotes fatty acids oxidation, which reduces lipid content in fish tissues.

3.2. The effects of dietary L-carnitine on blood biochemical parameters related to glucose metabolism and glycogen levels in tissues of Nile tilapia

Dietary L-carnitine elevated significantly the concentrations of glucose ($p = 0.033$; Fig. 3A), pyruvate ($p = 0.002$; Fig. 3B) and lactate ($p = 0.001$; Fig. 3C) in serum of treated fish compared to control. In addition, the carnitine supplementation increased significantly glycogen content in treated fish muscle ($p = 0.002$; Fig. 3E) compared to control, while it did not affect glycogen content in liver ($p = 0.721$; Fig. 3D). The elevation of glycogen accumulation in muscle as a response to dietary carnitine supplementation was further confirmed by histological analysis (Fig. 3F). These results indicate that L-carnitine attenuates glucose utilization and increases glycogen deposition in fish.

3.3. The effects of dietary L-carnitine on biochemical characteristics associated with protein metabolism in Nile tilapia

Dietary carnitine supplementation did not affect the concentration of total AAs in serum between treated and control fish ($p = 0.530$; Fig. 4A). However, L-carnitine supplementation significantly enhanced the total protein content in the carcass ($p = 0.001$; Fig. 4B) compared to control, but not in the whole body ($p = 0.800$; Fig. 4C). These data demonstrate that dietary L-carnitine promotes protein deposition in fish carcass, mainly in the muscle.

3.4. The effects of dietary L-carnitine on the activities of digestive enzymes in intestine of Nile tilapia

The fish fed on the L-carnitine supplemented diet significantly increased the activities of amylase ($p = 0.000$; Fig. 5B) and protease ($p = 0.027$; Fig. 5C) in intestine compared to control. However, L-carnitine supplementation did not affect lipase activity between the two groups of fish ($p = 0.316$; Fig. 5A). These findings indicate that L-carnitine has positive effects on the activities of intestinal digestive enzymes related to digestion and absorption of carbohydrate and protein.

3.5. The effects of dietary L-carnitine on the efficiency of oxidation, retention and conversion of macronutrients in Nile tilapia via isotope-based metabolic tracking

To test whether dietary L-carnitine supplementation directly affected catabolism of lipid, protein and glucose in fish, we performed a metabolic tracking test of macronutrients by i.p. injecting individual fish with ^{14}C-labeled (PA), glucose (Glu) and amino acids (AAs) mixture. After [1-^{14}C]-PA injection, the fish fed on L-carnitine supplemented diet increased significantly $^{14}CO_2$ release from [1-^{14}C]-PA oxidation ($p = 0.041$; Fig. 6A) compared to the control. Furthermore, the fish fed on L-carnitine supplemented diet showed relatively reduced

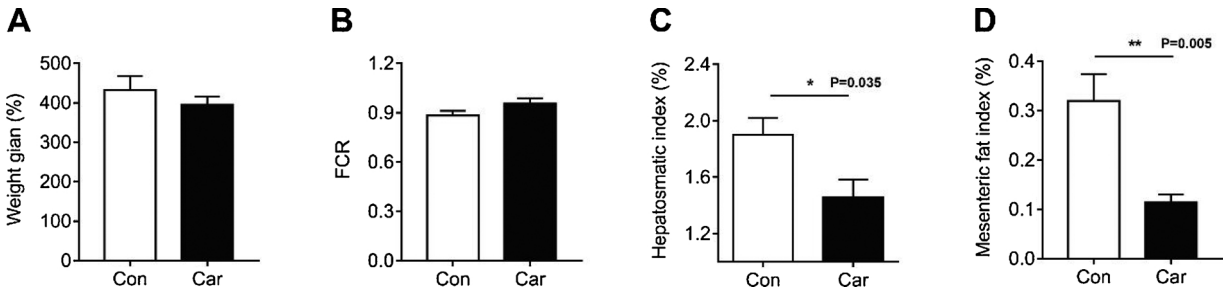


Fig. 1. The effect of dietary L-carnitine supplementation on growth, hepatosomatic index, and mesenteric fat index of Nile tilapia during the 8-week trial. (A) Weight gain (WG) ($n = 3$); (B) Feed conversion ratio (FCR) ($n = 3$); (C) Hepatosomatic index (HSI) ($n = 8$); (D) Mesenteric fat index (MFI) ($n = 8$). Con, control diet; Car, carnitine diet. *$p \leq 0.05$, **$p \leq 0.01$. Results are shown as means ± SEM.

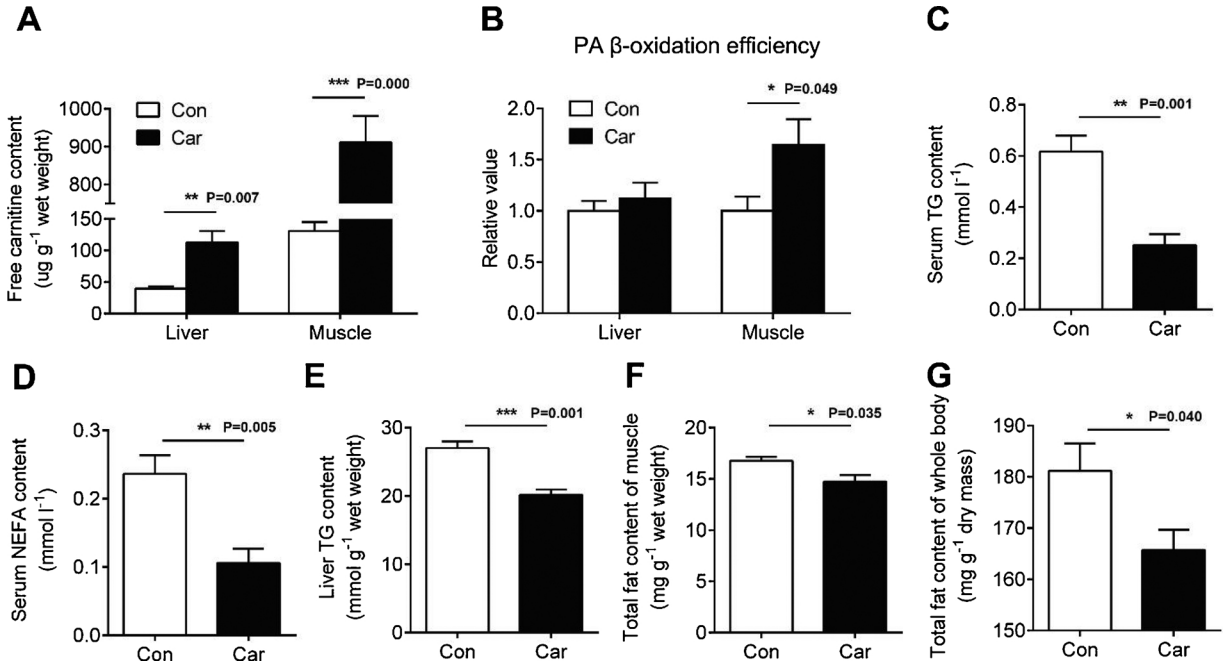


Fig. 2. The effect of dietary L-carnitine supplementation on lipid metabolic characteristics of Nile tilapia during the 8-week trial. (A) Free carnitine content in muscle and liver (n = 8); (B) The β-oxidation of [1-^{14}C]-palmitic acid (PA) in the homogenates of liver and muscle (n = 12); (C) Serum triglyceride (TG) content (n = 6); (D) Serum nonesterified free fatty acid (NEFA); (n = 6); (E) Hepatic TG content (n = 6); (F) Total fat content of muscle (n = 6); (G) Total fat content in whole body (n = 6). Con, control diet; Car, carnitine diet. *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001. Results are shown as means ± SEM.

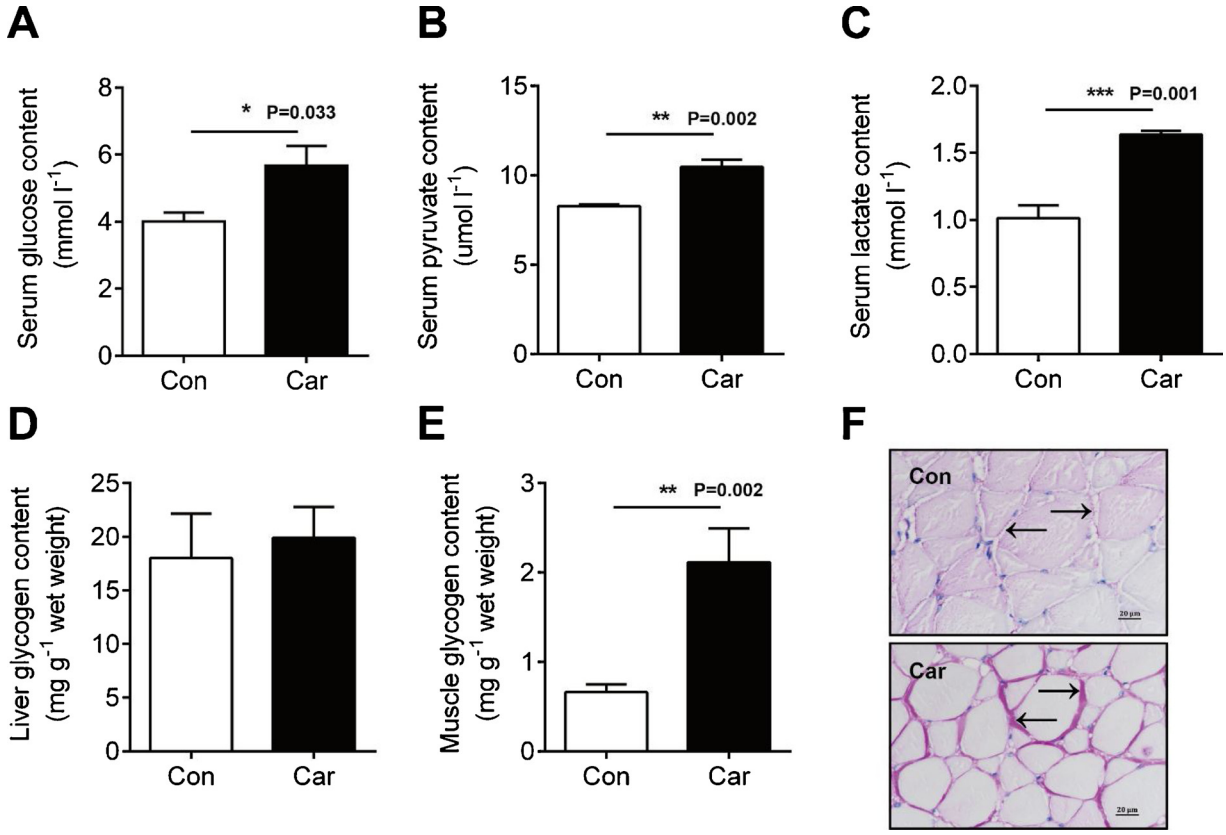


Fig. 3. The effect of dietary L-carnitine supplementation on glucose metabolic characteristics of Nile tilapia during the 8-week trial. (A) Serum glucose content (n = 6); (B) Serum pyruvate content (n = 6); (C) Serum lactate content (n = 6); (D) Hepatic glycogen content (n = 6); (E) Muscle glycogen content (n = 6); (F) PAS staining of muscle. Arrow shows the stained fuchsia glycogen granule in muscle cells. Con, control diet; Car, carnitine diet. *$p \leq$ 0.05, **$p \leq$ 0.01, ***$p \leq$ 0.001. Results are shown as means ± SEM.

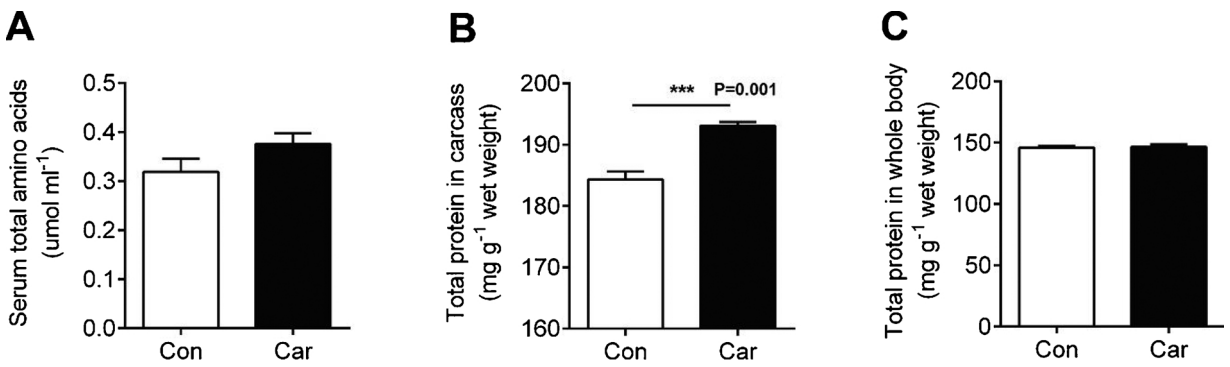


Fig. 4. The effect of dietary L-carnitine supplementation on protein metabolic characteristics of Nile tilapia during the 8-week trial. (A) Serum concentration (n = 6); (B) Total protein content of carcass (n = 8), the carcass of fish represents the body part without head, fins, tail and viscera; (C) Total protein content in whole body (n = 6). Con, control diet; Car, carnitine diet. ***$p \leq$ 0.001. Results are shown as means ± SEM.

trend of contents of the reserved ^{14}C-lipid (p = 0.799), ^{14}C-glycogen (p = 0.377) and ^{14}C-protein (p = 0.204) in the body without significant difference (Fig. 6B). During the D-[1-^{14}C]-Glu injection test, the fish fed on L-carnitine supplemented diet had higher release of $^{14}CO_2$ (11.88 % vs 9.16 %; p = 0.050) and lower proportion of ^{14}C-glycogen (8.74 % vs 11.38 %; p = 0.011) than the control (Fig. 6C and Fig. 6D). After the L-[^{14}C (U)]-AAs injection, the fish fed on L-carnitine supplemented diet had higher release of $^{14}CO_2$ from L-[^{14}C (U)]-AAs than the control (0.80 % vs 0.34 %; p = 0.039) (Fig. 6E). Although there was no significant difference in proportion of ^{14}C-glycogen (1.45 % vs 1.60 %; p = 0.330) and ^{14}C-protein (19.39 % vs 19.06 %; p = 0.848) between fish fed on L-carnitine diet and control, smaller proportion of ^{14}C-lipid was found in the fish fed on L-carnitine diet than control (7.48 % vs 10.77 %; p = 0.090) (Fig. 6F). There were no significant differences between Car and Con fish in the intermediate metabolites and metabolism products secreted in water after these nutrients injections (Fig. 6B, 6D and 6 F). All the above data indicate that dietary L-carnitine increases catabolism of lipid, glucose and AAs, when these nutrients are supplied as a single source.

4. Discussion

Recently, some studies have shown that dietary L-carnitine supplementation in fish regulates feed efficiency to increase growth performance, reduces body fat and modulates energy metabolism to provide a "protein sparing effect" (Harpaz, 2005; Li et al., 2019). However, these effects remain controversial in different fish species. In the present study, insignificant growth-promoting effect of L-carnitine supplementation was obtained in Nile tilapia, consistent with previous results in tilapia species (Du et al., 2002; Yang et al., 2009; Erdogan et al., 2015). Meanwhile, dietary L-carnitine supplementation reduced lipid content in muscle and whole body of Nile tilapia, in agreement with previous studies in channel catfish (Burtle and Liu, 1994), hybrid tilapia (Huang et al., 2010), Atlantic salmon (Ji et al., 1996), black carp (Ming et al., 2013), cobia (Wang et al., 2007), black sea bream (Ma et al., 2008), Japanese flounder (Gao et al., 2012), and zebrafish (Li et al., 2017). In contrast, previous studies reported that diets supplemented with L-carnitine reduce mesenteric fat ratio (Ozório et al., 2003; Yang et al., 2009, 2012) and lipid content in liver (Ozório et al., 2001; Du et al., 2002; Erdogan et al., 2015; Gou et al., 2016). The inconsistent results are probably due to tissue-specific effects of carnitine on various fish species and differences in nutrient composition of the feeds (Li et al., 2019). In the present study, the addition of L-carnitine in diets increased carnitine concentrations in both liver and muscle tissues, similar to many other reports (Ozório et al., 2001; Yang et al., 2012; Zheng et al., 2014; Li et al., 2017), indicating that Nile tilapia efficiently absorbs dietary carnitine. However, dietary L-carnitine supplementation was only found to accelerate significantly PA β-oxidation in muscle tissues, but not in the liver. Given that the endogenous biosynthesis of L-carnitine occurs mainly in the liver (Bremer, 1983; Li et al., 2019), liver might be the main target tissue for dietary L-carnitine supplementation in some fish species. However, in most animals, including fish, more than 95 % of the body's total carnitine stores occur in skeletal muscle tissue, where carnitine performs the translocation of LCFAs from cytosol into the mitochondria for β-oxidation (Stephens et al., 2007). Therefore, catabolism of lipids in muscle may also be significantly affected by L-carnitine in some fish species. It should be noted that the detailed information pertaining to carnitine synthesis and turnover in liver and muscle in most fish species are still unknown. Therefore, based on the present results, it is reasonable to conclude that both muscle and liver are the target tissues affected by dietary L-carnitine supplementation, because the concentration of L-carnitine in both

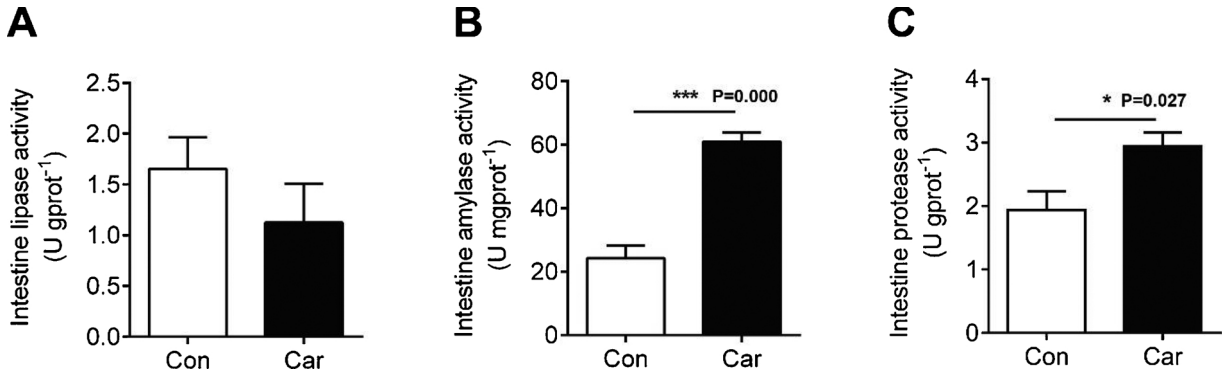


Fig. 5. The effect of dietary L-carnitine supplementation on intestine digestive enzymes activity of Nile tilapia during the 8-week trial. (A) Intestine lipase activity (n = 6); (B) Intestine amylase activity (n = 6); (C) Intestine protease activity (n = 6). Con, control diet; Car, carnitine diet. *$p \leq$ 0.05, ***$p \leq$ 0.001. Results are shown as means ± SEM.

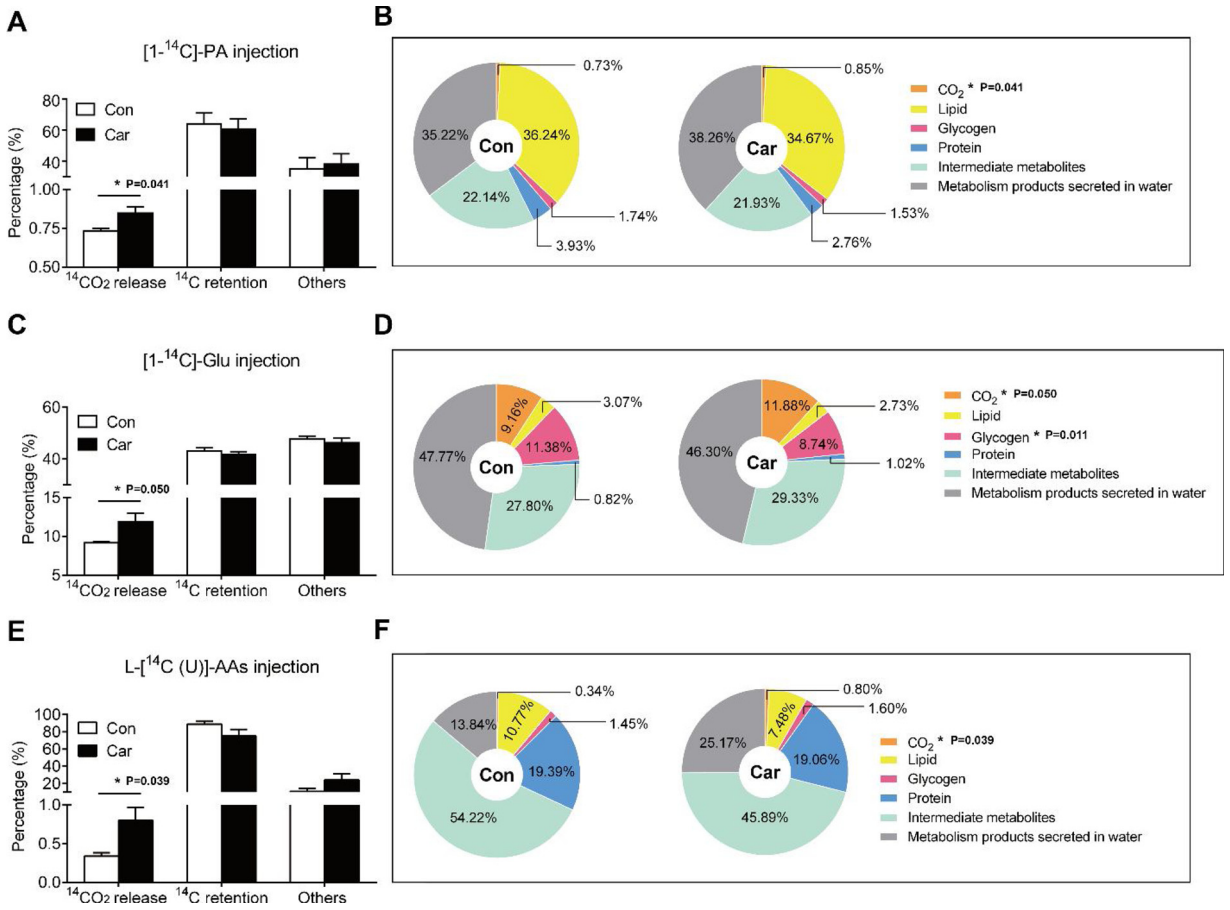


Fig. 6. The effect of dietary L-carnitine supplementation on the efficiency of oxidation, retention and conversion of macronutrients of Nile tilapia during the 8-week trial. (A, B) Metabolic tracking test of [1-^{14}C]-palmitic acid (PA) i.p. injected after 90 min (n = 6). (C, D) Metabolic tracking test of D-[1-^{14}C]-glucose (Glu) i.p. injected after 90 min (n = 6). (E, F) Metabolic tracking test of L-[^{14}C (U)]-amino acids mixture (AAs) i.p. injected after 90 min (n = 6). "Intermediated metabolites" represents the radioactive nutrients that are not been extracted from whole body. "Others" represents the metabolism products secreted in water during 90 min of labeled-nutrients injection. Con, control diet; Car., carnitine diet. *p ≤ 0.05. Results are shown as means ± SEM.

tissues was largely affected by dietary supplementation. However, the metabolic consequences of dietary carnitine supplementation vary among tissues in different fish species.

It is well known that, fish have lower ability to use carbohydrates as an energy source compared to mammals (Polakof et al., 2012). However, fish have higher ability to use protein as a source of energy; therefore, fish require higher dietary protein contents (Wilson, 1994). Previous studies have indicated that dietary L-carnitine supplementation in fish improves lipid catabolism for energy supply to provide a "protein sparing effect", resulting in protein deposition in muscle and whole body (Harpaz, 2005; Li et al., 2018). In most animals, including fish, nutrient metabolism is precisely regulated to maintain energy homeostasis (Murphy and Bloom, 2006; Condesieira and Soengas, 2017), by balancing the energy production sourced from lipid, carbohydrate and protein (Gale et al., 2004; Stengel et al., 2013). Therefore, when lipid-sourced energy is increased, the energy sourced from other nutrients, such as carbohydrate and protein may be decreased. We previously showed that dietary L-carnitine supplementation enhanced lipid catabolism by elevating mitochondrial fatty acid β-oxidation, and simultaneously caused a decrease in glucose utilization and promoted protein synthesis in zebrafish (Li et al., 2017). In contrast, our recent study showed that in the low-carnitine zebrafish model, glucose oxidation rate was elevated, and body glycogen content was decreased, accompanied with an enhanced glucose uptake and glycolysis (Li et al., 2018). In the present study, dietary L-carnitine also reduced carbohydrate utilization in Nile tilapia. The fish fed on L-carnitine supplemented diet increased serum glucose content by 1.5-fold compared to control, suggesting that the carnitine-fed fish had lower glucose disposal capacity, consistent with the results in Japanese flounder (Gao et al., 2012), common carp (Sabzi et al., 2017) and silver perch (Yang et al., 2012). Moreover, L-carnitine-elevated the glycogen deposition in Nile tilapia muscles due to reduced glucose catabolism, which is consistent with the previous results that carnitine-fed zebrafish elevated whole body glycogen storage, upregulated gluconeogenesis and downregulated glycolysis (Li et al., 2017). Therefore, dietary carnitine-improved lipid catabolism results in decreased glycolysis and increased gluconeogenesis and glycogen synthesis. These effects of L-carnitine on the carbohydrate metabolic pathway may decrease the energy portion sourced from glucose utilization and induce glycogen deposition in fish. It should be noted that, dietary L-carnitine also increased the activity of intestinal amylase, suggesting an elevated digestion and absorption of carbohydrate from diet, which is consistent with increased serum glucose concentration. However, the detailed mechanism is still waiting for further investigations.

Similarly, L-carnitine also increased protease activity and protein content in the carcass of Nile tilapia, which is similar to the results obtained in other fish species (Zhang et al., 2009; Gao et al., 2012; Sharifzadeh et al., 2017). Previously, we indicated that dietary L-carnitine increased the whole body protein content in zebrafish by upregulating the mechanistic target of rapamycin (mTOR) gene expression (Li et al., 2017). The mTOR is one of the most important regulatory elements of protein synthesis (Wang and Proud, 2006); therefore, dietary supplementation with L-carnitine might have positive effects on protein synthesis in Nile tilapia. Therefore, our present data and

Table 2
Amino acids levels of experimental diets.

Amino acids (g/kg)	Control (Con)	L-carnitine (Car)
Asp	27.38	26.61
Thr	14.63	14.19
Ser	18.81	18.35
Glu	76.65	74.72
Gly	23.73	22.84
Ala	16.42	16.45
Val	20.80	20.72
Met	9.43	9.26
Ile	16.16	15.65
Leu	30.65	29.76
Tyr	16.48	15.82
Phe	16.82	16.48
Lys	26.85	26.26
His	8.65	8.45
Arg	16.26	15.95

Asp, asparagine; Thr, threonine; Ser, serine; Glu, glutamate; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Try, tryptophane; Phe, phenylalanine; Lys, lysine; His, Histidine; Arg, arginine.

previous literature indicate that dietary L-carnitine supplementation promotes protein deposition in fish, possibly because L-carnitine increases lipid-sourced energy supply thereby inhibits protein catabolism for energy consumption. On the other hand, L-carnitine is synthesized from essential AAs, lysine and methionine (Bremer, 1983; Li et al., 2019). The levels of lysine (Lys) and methionine (Met) in the control diet were 26.85 g/kg and 9.43 g/kg (Table 2), respectively, which meet the optimal amino acids requirement for tilapia growth as reported previously (Santiago and Lovell, 1988; He et al., 2013). Therefore, dietary L-carnitine supplementation may spare these essential AAs and promote protein synthesis as previous discussed (Li et al., 2019). In Atlantic salmon, dietary L-carnitine supplementation increased accumulation of protein in organs, and the concentrations of free AAs such as leucine, isoleucine, and valine in plasma and liver (Ji et al., 1996). Leucine has been proved to stimulate effectively mTOR signaling (Kimball and Jefferson, 2006; Lansard et al., 2011; Son et al., 2018), resulting in the promotion of protein synthesis (Wang and Proud, 2006; Saxton and Sabatini, 2017). Therefore, L-carnitine might also increase protein deposition through leucine-stimulated mTOR signaling pathway, an observation that calls for further studies. In the present study, increased intestinal protease activity may also suggest that the protein digestion was elevated to supply AAs for the strengthened *in vivo* protein synthesis.

However, different from our present data and above literature, some studies showed that dietary L-carnitine supplementation had no significant impact on protein content in body and muscle of fish (Li et al., 2010; Erdogan et al., 2015; Li et al., 2019), and some studies even reported that dietary L-carnitine had negative effects on protein accumulation (Kordi et al., 2012). Therefore, the ability of L-carnitine to play different roles in protein deposition in various studies remains an important question for fish nutritionists. This question prompted us to perform the metabolic tracking of different macronutrients in the fish fed on L-carnitine.

A number of researches have shown that the effects of L-carnitine supplementation on protein deposition may be influenced by several factors, including feed composition such as lipid or protein levels (Torreele et al., 1993; Chen et al., 2010), methionine and lysine contents (Chatzifotis et al., 1996; Chen et al., 2011; Gou et al., 2016) and vitamin C levels (Sharifzadeh et al., 2017). However, it also remains unknown whether L-carnitine could only stimulate catabolism of lipid or may also elevate breakdown of glucose and protein. Therefore, in the present study, we performed the metabolic tracking test of macronutrients by i.p. injecting individual fish with ^{14}C-labeled PA, Glu and AAs. The results showed that L-carnitine undoubtedly increased the content of $^{14}CO_2$ release from [1-^{14}C]-PA oxidation and decreased the retention rate of ^{14}C-nutrients in the body when a single fatty acid was supplied, as previously reported (Ning et al., 2016). These data suggest that when carnitine-fed fish were given a single fatty acid as a nutrient, the ingested fatty acid was preferentially oxidized but neither esterified nor converted to other nutrients. When the ^{14}C-labeled Glu or AAs was injected, we found dietary L-carnitine supplementation also increased the $^{14}CO_2$ release from the D-[1-^{14}C]-Glu and L-[^{14}C (U)]-AAs, respectively. Moreover, after the D-[1-^{14}C]-Glu injection, the carnitine-fed fish had lower proportion of ^{14}C-glycogen deposition. These results indicate that when Glu and AAs are supplied as single nutrients, respectively, the oxidation rate of nutrient could be elevated in the carnitine-fed fish for energy generation. The present study indicates, for the first time, that when lipid supply is not adequate, dietary L-carnitine supplementation can also increase the catabolism of glucose and AAs as energy source. From these results, we demonstrate that the effects of dietary L-carnitine supplementation on nutrient metabolism are tightly correlated with the relative abundance of dietary macronutrients. When the diet contains abundant lipid, L-carnitine supplementation preferably triggers mitochondrial fatty acid β-oxidation to produce lipid-sourced energy efficiently and simultaneously weakens catabolism of glucose and protein to lower glucose- and/or protein-sourced energy production. Therefore, dietary L-carnitine supplementation may increase glycogen and protein deposition, especially when dietary lipid is abundant. On the contrary, when lipid substrate is insufficient, L-carnitine may possibly improve glucose or protein oxidation, thus decreases glycogen and protein deposition in body. In the present study, we fed fish with the diets containing 6.6 % lipid, 30 % carbohydrate and 31.7 % protein. The optimal dietary lipid content of Nile tilapia has been reported to range from 5 to 7.4 % (He et al., 2015; Ning et al., 2016), thus 6.6 % dietary lipid in the present study was sufficient to cause the “protein sparing effect” of L-carnitine. However, L-carnitine supplementation had no significant changes in the protein content in red sea bream (Chatzifotis et al., 1996), Nile tilapia (Erdogan et al., 2015) and large yellow croaker (Sang et al., 2012), which were supplemented with low levels of dietary lipid, and even caused negative effects on protein deposition in Caspian roach (Kordi et al., 2012) and beluga sturgeon (Mohseni et al., 2008). Accordingly, if dietary lipid is not adequate, dietary L-carnitine supplementation may increase catabolism of glucose and protein as observed in the present study, and the “protein sparing effect” may not be obtained.

5. Conclusion

The results of the present study indicated that dietary L-carnitine supplementation efficiently stimulate lipid catabolism and increase glycogen and protein deposition in Nile tilapia muscle. However, when fatty acid, glucose and amino acids were provided individually as single nutrients, dietary L-carnitine supplementation elevated catabolic efficiency of each macronutrient. These results suggest that the effects of dietary L-carnitine on nutrient metabolism are correlated with the abundance of individual macronutrients, and the “protein-sparing effect” of L-carnitine may be obtained when diets contain adequate lipid content. The present study provides a new insight to the regulation mechanism of L-carnitine on nutrient metabolism in fish.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution

L.-Y. L. and Z.-Y. D. designed the experiment. L.-Y. L., D.-L. L. and Z.-Y. J. conducted the experiment and sample analysis. L.-Y. L., Z.-Y. J. and F. Q. analyzed the data. M.-L. Z. and L.-Q. C. provided essential technical supports. L.-Y. L. drafted the manuscript. S. M. L. and Z.-Y. D. revised the manuscript.

Declaration of Competing Interest

Authors declare that they have no conflict of interest in this work.

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