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Effect of Dietary L-Carnitine Supplementation on Growth, Serum Biochemical Indices, Oxidative and Growth-Related Gene Expressions in Chinese Soft-Shelled Turtle (*Pelodiscus sinensis*)

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XL and HL designed the study and prepared the manuscript. XL, MAOD, FW, WL, JT, MJ and LY performed the experiments and analyzed the data. HW designed the experiments. XL and NX provided the experimental animals.

Key words
***Pelodiscus sinensis*, L-carnitine, Growth performance, Lipid deposition, Gene expression**

ABSTRACT

This study was conducted to investigate the effects of dietary L-carnitine supplementation in Chinese soft-shelled turtle, *Pelodiscus sinensis*. Four experimental diets were formulated with the addition of L-carnitine at the levels of 0 (control), 100, 200 and 400 mg kg^{-1}. Each diet was fed to triplicate groups of turtles with initial average weight (394.4 ± 3.8 g) for 8 weeks. Our results showed that the weight gain rate (WGR), specific growth rate (SGR) and feed efficiency (FE) of Chinese soft-shelled turtles increased linearly with increasing dietary L-carnitine levels from 0 to 200 mg kg^{-1}, but declined slightly with the L-carnitine supplementation at 400 mg kg^{-1}. Muscle protein content was significantly increased when turtles were fed diets with 200 or 400 mg kg^{-1} L-carnitine ($P < 0.05$). The serum biochemical indices analysis revealed that dietary L-carnitine at 400 mg kg^{-1} had markedly reduced triglyceride (TG) concentration ($P < 0.05$). The elevated expression of hepatic *igf1* gene at the transcriptional level was positively correlated with dietary L-carnitine levels on the growth performance of Chinese soft-shelled turtle. Furthermore, dietary L-carnitine supplementation significantly up-regulated the mRNA levels of superoxide dismutase 1 (*sod1*) and lipoprotein lipase (*lpl*) genes in the liver of Chinese soft-shelled turtle ($P < 0.05$). All the above results indicated that appropriate dietary L-carnitine supplementation could improve growth performance, increase muscle protein content, reduce serum TG concentration, and induce the antioxidant- and lipometabolism-associated genes expression in Chinese soft-shelled turtle.

INTRODUCTION

L-carnitine (β-OH-γ-*N, N, N*-trimethylaminobutyric acid) is a small compound that is synthesized in vivo from lysine and methionine with the assistance of vitamin C and other secondary substrates generated in the body (Harpaz, 2005). It plays important roles in lipid metabolism and energy production by transporting long-chain fatty acids into the inner mitochondrial matrix for β-oxidation and subsequent oxidative phosphorylation (Dayanandan *et al*., 2001; Zheng *et al*., 2014). Generally, L-carnitine was used as a bioactive and non-pollution additive for fish cultivation (Mohseni *et al*., 2008). Numerous studies have demonstrated that dietary supplementation with moderate concentrations of L-carnitine can be beneficial for increasing growth rate and decreasing body lipid content in some species such as African catfish, *Clarias gariepinus* (Ozório *et al*., 2001), hybrid striped bass, *Morone saxatilis* × *Morone chrysops* (Twibell and Brown, 2000), and silver perch, *Bidyanus bidyanus* (Yang *et al*., 2012). Moreover, L-carnitine has been found to act as an antioxidant to reduce lipid peroxidation in black sea bream, *Sparus macrocephalus* (Ma *et al*., 2008) and beluga, *Huso huso* (Mohseni and Ozório, 2014). Thus, dietary L-carnitine supplements in aquaculture are essential in terms of improvement in growth performance, antioxidant status and lipid metabolism.

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The Chinese soft-shelled turtle, *Pelodiscus sinensis*, is a high-valued freshwater species that is widely distributed in central and southern China and south-eastern Asia (Wu *et al*., 2013). This animal has become one of the favorite aquaculture species in China due to its excellent nutritional and medicinal values (Huang and Lin, 2004). In 2014, soft-shelled turtle production reached over 341,000 tons in China (Xu *et al*., 2016). However, owing to the intensive cultivation in some turtle farms, excessive lipid deposition and poor antioxidant capacity in soft-shelled turtle may affect the quality of final harvest. Consequently, how to improve the nutritional quality of soft-shelled turtle remains a major concern.

To our knowledge, there is limited information regarding the effects of L-carnitine on the Chinese soft-shelled turtle. The present study is designed to evaluate the effects of dietary L-carnitine supplementation on growth performance, muscle composition and serum total cholesterol (TCHO), triglyceride (TG) and total protein (TP) concentrations of soft-shelled turtle. In addition, the mRNA levels of genes in liver including insulin-like growth factor 1 (*igf1*), superoxide dismutase 1 (*sod1*) and lipoprotein lipase (*lpl*) were examined by quantitative real-time PCR.

MATERIALS AND METHODS

Experimental diets

Four experimental diets were formulated to contain L-carnitine at the graded levels of 0 (control), 100, 200 and 400 mg kg^{-1}. The ingredients and proximate analysis are shown in Table I. All experimental diets were produced according to the methods described by the previous study (Huang and Lin, 2004). Briefly, feed ingredients were mixed using a KitchenAid multi-function mixer and stored in a -20°C freezer. Before feeding, water and soybean oil were added to the feed powder in a 1:2 (v/w) ratio to produce fresh feed dough, and then cut it into 6-8 mm small pieces to feed the turtles. The feed dough for each group was prepared once every two days.

Experimental procedure

Chinese soft-shelled turtles were obtained from Anhui XIjia Agricultural Development Co. Ltd in Bengbu city, China. The animals were conditioned for 2 weeks and fed with the basal diet to adjust to the experimental diet. After acclimatization, apparently healthy turtles of uniform size (mean initial weight 394.4 ± 3.8 g) were selected and randomly assigned to 12 plastic tanks (*L* 90 cm × *W* 60 cm × *H* 35 cm). To prevent fighting, each tank was assigned to three turtles and there were nine turtles for each treatment. Four experimental diets containing different levels of L-carnitine were randomly allocated to 36 turtles. The height of the water in each tank was about the same as the height of the turtles to give the turtles room to breath. Turtles were maintained indoors at 32 ± 1 °C and fed the experimental diets three times daily (08:30, 13:30 and 18:30) at 1 % of average body weight for 8 weeks. This feed ration was chosen based on our observation during the acclimation period and was the amount of feed that turtles could consume in 20-30 min. The uneaten food was removed 30 min after feeding and measured to calculate feed intake. Fecal matter was siphoned from the bottom of each tank at 20:00, and equivalent water was supplemented into the tank. Water quality was monitored in the morning once every three days at 07:00. All turtles were weighed every two weeks. The daily feed quantities were adjusted according to the measurement.

Table I. Composition and proximate analysis of experimental diets. Data is given in % (on the basis of dry weight).

	Groups/ dietary L-carnitine levels (mg kg^{-1} diet)			
	0 (control)	**100**	**200**	**400**
Ingredients				
White fish meal	55.00	55.00	55.00	55.00
Soybean meal	10.00	10.00	10.00	10.00
α-starch	23.00	23.00	23.00	23.00
Beer yeast	5.00	5.00	5.00	5.00
Corn oil	3.00	3.00	3.00	3.00
Vitamin premix [1]	0.50	0.50	0.50	0.50
Mineral premix [2]	1.00	1.00	1.00	1.00
Choline chloride	0.25	0.25	0.25	0.25
Calcium biphosphate	1.25	1.25	1.25	1.25
L-carnitine [3]	0	0.25	0.50	1.00
Cellulose	1.00	0.75	0.50	0
Proximate composition				
Moisture	8.51	8.34	8.31	8.29
Crude protein	42.91	43.49	43.03	42.51
Crude lipid	4.18	4.19	4.75	4.13
Ash	14.23	14.45	14.01	14.53

[1]Vitamin premix consisted of (IU or g kg^{-1} premix): vitamin A, 8 IU; vitamin D, 1 IU; vitamin E, 80; vitamin K_3, 3; thiamine, 4; riboflavin, 4; pyridoxine, 8; cyanocobalamin, 2; nicotinic acid, 32; calcium pantothenate, 40; folic acid, 2; biotin, 6; vitamin C, 60; inositol, 100. [2]Mineral premix consisted of (g kg^{-1} premix): Na_2SeO_3, 0.4; $CaCO_3$, 350; $NaH_2PO_4 \cdot H_2O$, 200; KH_2PO_4, 200; $MgSO_4 \cdot 7H_2O$, 100; $MnSO_4 \cdot H_2O$, 2; $CuCl_2 \cdot 2H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 10; $FeSO_4 \cdot 7H_2O$, 10; NaCl, 120; KI, 0.1; $CoCl_2 \cdot 6H_2O$, 2; $AlCl_3 \cdot 6H_2O$, 1. [3]L-carnitine was purchased from Xi'an Hao Yuan Bio Technology Co., Ltd.

Sample collection and analysis

At the end of the 8-week period, all turtles were deprived of food for approximately 24 h before weighing. Survival rate (SR), weight gain rate (WGR), specific growth rate (SGR) and feed efficiency (FE) were calculated. After determining the final total weight of the turtles in each tank, three turtles per tank were selected and killed to obtain liver and muscle samples. The liver tissue was weighed to measure the hepatosomatic index (HSI). Blood samples were collected from the head vein of the turtle and centrifuged for separation of serum (2,500 g, 10 min). All samples (muscle, liver, and serum) were stored at -80 °C until analyzed.

Proximate composition of the experimental diet and muscle samples was determined using standard methods (AOAC, 2005). For moisture content determination, diet and muscle samples were dried at 105°C to a constant weight. Crude protein content was measured by the Kjeldahl method after an acid digestion using an auto Kjeldahl System (kjelflex K360; BUCHI, Flawil, Switzerland). Lipid content was determined by Soxhlet ether-extraction. Ash content was determined using a muffle furnace at 550°C for 24 h.

Concentrations of serum TCHO, TG and TP were all measured with an Automatic Biochemical Analyzer (Sysmex-800, Sysmex Corporation, Kobe, Japan) using commercial diagnostic reagent kits (Shanghai Sysmex Medical Electronics Corporation, Shanghai, China).

RNA extraction and quantitative real-time PCR

Total RNAs were extracted from liver tissue of turtles in each tank using TRIZOL reagents (Invitrogen, USA) according to the manufacturer's recommendations. RNA samples were digested with RNase-free DNase I (Takara, China) for 30 min at 37 °C to remove residual DNA. The RNA quality was then assessed with agarose gel electrophoresis and UV spectrophotometry. The cDNAs were transcribed from 4 μg of total RNA using the RevertAid™ First-Strand cDNA Synthesis Kit from Fermentas.

Quantitative real-time PCR was carried out on an Applied Biosystems 7500 Real-Time PCR System with SYBR Green Real Master Mix from Tiangen Bio, China. The expression of *β*-actin was used as the internal reference for gene normalization. Primers for *igf1*, *sod1*, *lpl* and *β*-actin were designed by the Primer Premier 6.0 software and are listed in Table II. All qRT-PCRs were performed in a 20-μL volume. The thermocycling conditions for the reaction were as follows: 95°C for 5 min, followed by 40 cycles consisting of 95°C for 10s, 60°C for 30s, and 72°C for 20s. The reaction was run in triplicate for each sample. Data was expressed as the relative expression of the reference gene using the $2^{-\Delta\Delta Ct}$ method (Lu *et al*., 2014).

Statistical analysis

All data was presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were performed using SPSS version 18.0 to determine the significant difference ($P < 0.05$) among different treatments.

RESULTS

Survival and growth performance

After an 8-week feeding trail, all turtles were in good health and no mortality was observed in any group. Growth performance and feed utilization of soft-shelled turtles fed experimental diets with different L-carnitine levels are shown in Table III. WGR and SGR of soft-shelled turtles increased with increasing dietary L-carnitine levels from 0 to 200 mg kg^{-1} but declined when the L-carnitine supplementation was at 400 mg kg^{-1}. Turtles fed diets supplemented with 200 mg kg^{-1} L-carnitine had a higher WGR, SGR and FE than those fed diets with 0 (control), 100 and 400 mg kg^{-1} L-carnitine ($P < 0.05$). The HSI was the highest for turtles fed the diet supplemented with 200 mg kg^{-1} L-carnitine, but it was not significantly different among the other treatments.

Muscle composition

Muscle composition of soft-shelled turtles fed diets with various levels of L-carnitine for 8-week are presented in Table III. Crude protein content of muscle increased with the dietary L-carnitine supplementation; it reached the highest in the 200 mg kg^{-1} L-carnitine-supplement group ($P < 0.05$). In contrast, crude lipid content declined with increasing dietary L-carnitine levels, although no significant differences were observed in these four groups. In addition, dietary L-carnitine supplementation did not affect muscle moisture and ash content.

Serum biochemical indices

Some serum biochemical indices of soft-shelled turtles fed diets with different L-carnitine levels for 8-weeks are listed in Table III. Serum TCHO and TG concentrations tended to decrease with increasing dietary L-carnitine levels, and the lowest TCHO and TG concentrations were all observed in turtles fed diet supplemented with 400 mg kg^{-1} L-carnitine. It is noted that TG concentration was significantly lower in turtles fed with 400 mg kg^{-1} L-carnitine than that in turtles fed the control diet ($P < 0.05$). However, there were no significant differences in serum TP concentration among four treatment groups.

Table II. Nucleotide sequences of primers used in quantitative real-time PCR.

NCBI Reference sequence	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)
NM_001286920	*igf1*	ATGCTGCTTCCAGAGCTGTGAC	CAGTGTGGCGTTGAGCACGTA	101
JX470524	*sod1*	CGGTGAAGGGCGTCATCAACTT	GAGCACCTGCACTGGTACATCC	147
XM_006111116	*lpl*	CATCCAGCGTGTCCGAGTGAAG	ACACTGCCGCCTCCTTTCCT	104
XM_006112915	*β-actin*	TGATGGACTCAGGTGACGGTGT	GGCTGTGGTGGTGAAGCTGTAG	158

Table III. Effect of dietary L-carnitine levels on growth performance, muscle composition and serum index of Chinese soft-shelled turtles fed experimental diets for 8 weeks*.

	Groups/dietary L-carnitine levels (mg kg^{-1} diet)			
	0 (control)	**100**	**200**	**400**
Growth performance				
IBW (g)	394.93±3.97	395.40±4.28	390.13±2.72	397.07±4.13
FBW (g)	466.97±4.38^{a}	476.33±5.51^{a}	503.33±12.50^{b}	466.67±7.64^{a}
WGR (%)	18.24±1.20^{a}	20.49±2.65^{a}	29.01±2.46^{b}	17.54±2.58^{a}
SGR (%/d)	0.30±0.02^{a}	0.33±0.04^{a}	0.46±0.03^{b}	0.29±0.04^{a}
FE	0.66±0.01^{a}	0.69±0.01^{b}	0.73±0.01^{c}	0.68±0.01^{ab}
HSI (%)	4.26±0.08	4.28±0.13	4.57±0.17	4.23±0.14
Muscle composition				
Moisture	77.40±0.61	77.13±0.75	77.25±0.30	78.32±1.14
Crude protein	13.61±0.55^{a}	14.85±0.98^{a}	17.35±0.85^{b}	17.30±1.09^{b}
Crude lipid	1.38±0.07	1.34±0.06	1.24±0.05	1.28±0.08
Ash	4.63±0.03	4.62±0.15	4.63±0.09	4.65±0.05
Serum index				
TCHO/mmol L^{-1}	7.63±0.65	7.40±0.69	7.22±0.35	7.20±0.52
TG/mmol L^{-1}	3.42±0.20^{b}	3.06±0.17^{ab}	2.52±0.26^{ab}	2.48±0.23^{a}
TP/g L^{-1}	34.67±2.08	32.67±1.16	33.24±1.41	33.50±2.12

*: Values are mean ± SD of the three replicate per treatment and values within the same row with different letter superscripts are significantly different at $P < 0.05$. IBW, initial mean weight; FBW, final mean weight; WGR (weight gain rate, %) = 100 × (FBW – IBW) / IBW; SGR (special growth rate, % d^{-1}) = 100 × (ln FBW– n IBW) / feeding days (d); FE (feed efficiency) = (g total final weight – g total initial weight + g dead turtle) / g feed intake; HSI (hepatosomatic index, %) = 100 × (g liver weight) / (g body weight).

Transcriptional expression levels of hepatic igf1, sod1 *and* lpl *genes*

The relative mRNA expression of growth-related gene *igf1*, antioxidant-associated gene *sod1,* and lipid metabolism-relevant gene *lpl* in the liver of soft-shelled turtles after 8-week of feeding with different L-carnitine levels was detected. As shown in Figure 1A and B, *igf1* and *sod1* mRNA levels both significantly increased with increasing dietary L-carnitine levels from 100 to 200 mg kg^{-1} ($P < 0.05$), but then returned to the control levels when the L-carnitine supplementation was at 400 mg kg^{-1}. The transcriptional expression of *lpl* was also significantly induced by dietary L-carnitine supplementation from 100 to 400 mg kg^{-1}, and the relative mRNA levels of *lpl* were 1.7, 3.0 and 2.4 times, respectively (Fig. 1C). Moreover, all of the highest expressions for corresponding *igf1*, *sod1* and *lpl* genes were observed in turtles fed with 200 mg kg^{-1} L-carnitine supplementation.

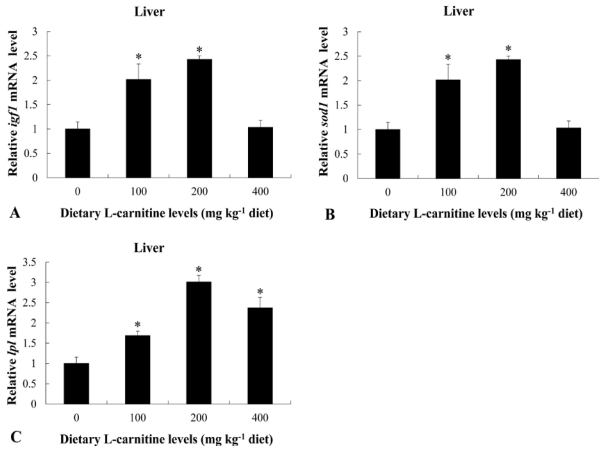

Fig. 1. Effect of dietary L-carnitine levels on the transcriptional expression of (A, B, C).

DISCUSSION

Growth performance

L-carnitine has been demonstrated to promote fatty acid oxidation by the mitochondria and increase the protein sparing action of fat, and thus lead to better growth in fish and crustacean species (Mohseni *et al*., 2008). In the present study, a growth-enhancing effect of the appropriate levels of L-carnitine supplementation was found in soft-shelled turtles, which is in agreement with results obtained in some marine and freshwater species (Becker *et al*., 1999; Chatzifotis *et al*., 1995; Ma *et al*., 2008; Santulli and d'Amelio, 1986; Torreele *et al*., 1993). Our results showed that within the experimental levels from 100 to 200 mg kg^{-1}, supplemental dietary L-carnitine exhibited positive effects in terms of FBW, WGR and SGR. In contrast, dietary L-carnitine at a concentration of 400 mg kg^{-1} did not significantly promote growth performance of soft-shelled turtles. Similar results in unchanged, or even reduced, weight gain caused by higher concentrations of L-carnitine were also found in previous studies (Keshavanath and Renuka, 1998; Zheng *et al*., 2014). For example, Zheng *et al*. (2014) reported that 331 mg kg^{-1} L-carnitine supplementation resulted in the highest FBW and WGR, while higher levels of supplementation were less effective in yellow catfish (*Pelteobagrus fulvidraco*). This could be attributed to energy loss through the excretion of excess acylcarnitine (Zheng *et al*., 2014). It should be pointed out that growth rates of soft-shelled turtles were increased as a result of improvement in feed utilization. The findings relating to growth and feed efficiency are consistent with those of Torreele *et al*. (1993), Yang *et al*. (2012) and Zheng *et al*. (2014).

Moreover, the relative expression of *igf1* mRNA in the liver of turtles fed with different L-carnitine levels was examined. The gene *igf1* is a growth-promoting polypeptide that is critically important for normal growth and development of this organism (Yakar *et al*., 2002). In this study, a significant increase in mRNA levels of hepatic *igf1* gene was found when turtles were fed with 100-200 mg kg^{-1} L-carnitine supplementation, in accordance with the results of increased WGR and SGR. Thus, considering the elevated expression of *igf1* gene at the transcriptional level, we concluded that dietary L-carnitine supplementation is beneficial for growth of Chinese soft-shelled turtles, and the adequate dietary L-carnitine to promote optimal growth is 200 mg kg^{-1}.

Muscle composition

In the present study, dietary L-carnitine supplementation was found to reduce lipid content but significantly increase protein content in the muscle of soft-shelled turtles, similar to that observed in previous studies in channel catfish (Burtle and Liu, 1994), Atlantic salmon (Ji and Bradley, 1996), rohu carp (Keshavanath and Renuka, 1998), black sea bream (Ma *et al*., 2008) and beluga (Mohseni and Ozório, 2014; Mohseni *et al*., 2008). However, several studies have shown that dietary L-carnitine could increase lipid content in the muscle of red sea bream (Chatzifotis *et al*., 1995) and rainbow trout (Selcuk *et al*., 2010). Recent work by Zheng *et al*. (2014) reported that diets with low and high L-carnitine supplements significantly improved lipid accumulation in the liver and muscle of yellow catfish. The variation in these results may be caused by various experimental conditions and species differences (Harpaz, 2005; Zheng *et al*., 2014).

Serum biochemical indices

Research has shown that in human and terrestrial animals, supplemental L-carnitine has a great effect on reducing serum total cholesterol (TCHO) and triacylglycerols (TG) concentrations (Bell *et al*., 1987; Rebouche, 1992). In fish, Santulli *et al*. (1988) and Mohseni and Ozório (2014) reported that the supplementation of dietary L-carnitine could decrease the serum TCHO concentrations in European sea bass and beluga, respectively. In this study, decreasing trends in serum levels of TCHO and TG were also found in Chinese soft-shelled turtles when fed with increasing dietary L-carnitine levels from 100 to 400 mg kg^{-1}. These results indicate that the lipid-lowering effects in blood was a result of L-carnitine administration. However, non-significant differences in the findings of the present study for serum total protein (TP) concentration were observed among all groups. This is probably due to the result of β-oxidation of long chain fatty acids with the support of

additional L-carnitine (Arslan *et al*., 2003).

Antioxidant and lipometabolism-associated genes

A large number of studies have suggested that L-carnitine could improve antioxidant functions by protecting against free radical damage and enhancing lipid metabolism through facilitating fatty acid oxidation in human and animals (Arduini, 1992; Gülçin, 2006; Hoppel, 2003; Pekala *et al*., 2011). The liver is the primary site for multiple oxidative reactions (Gül *et al*., 2004) and fatty acid synthesis (Xu *et al*., 2003). Thus, to detect effects of dietary L-carnitine on soft-shelled turtles, the transcriptional expression of antioxidant- and lipometabolism-related genes in liver was necessary to be investigated. Superoxide dismutase 1 (*sod1*) gene is reported to encode the enzyme copper zinc superoxide dismutase, which catalyzes the dismutation of superoxide radicals produced during biological oxidations and environmental stress (Park and Rho, 2002). For this reason, it is considered as an important indicator of oxidative stress in cells and tissues (Mishra *et al*., 2016). In this study, a significant increase of 2.1-2.4 fold in hepatic *sod1* mRNA levels was found when turtles were fed with 100 or 200 mg kg^{-1} L-carnitine, indicating that dietary L-carnitine supplementation could potentially improve the antioxidant capacity of soft-shelled turtle by increasing transcript level of antioxidant enzymes. Lipoprotein lipase (LPL) is encoded by *lpl* gene and known as a rate-limiting enzyme for the uptake of triacylglycerol (TG) derived fatty acids (FAs) (Tian *et al*., 2013). It could hydrolyze TG present in plasma lipoproteins and supply non-esterified fatty acids for storage, or β-oxidation in tissues and play a pivotal role in the regulation of lipid synthesis. Furthermore, LPL has been found to promote the uptake of lipoproteins by the liver in fish (Liang *et al*., 2002). In the present study, *lpl* mRNA levels were significantly induced by dietary L-carnitine supplementation at concentrations from 100 to 400 mg kg^{-1}, suggesting this lipogenic enzyme was mainly regulated by L-carnitine at the transcriptional level. On the other hand, the increase in *lpl* transcripts might indicate an increase in the import of lipids into the liver for fatty acid synthesis and support a decline in blood TG and lipid consumption in the muscle of soft-shelled turtle. This data is highly consistent with the previous report by Zheng *et al*. (2014). However, the specific molecular mechanism by which L-carintine regulated the expression of liver *sod1* and *lpl* gene remains to be studied.

CONCLUSION

Our results show that adequate dietary L-carnitine supplementation has positive effects on the growth of Chinese soft-shelled turtle. Supplemental dietary L-carnitine may also improve the meat quality of Chinese soft-shelled turtle by reducing muscle lipid deposition and regulating blood lipid levels. Moreover, the transcriptional expression of hepatic *sod1* and *lpl* genes in Chinese soft-shelled turtle were significantly induced by dietary L-carnitine levels, which can provide information on diet utilization and be used as molecular markers of the nutritional status for Chinese soft-shelled turtle.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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