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Myo-inositol improves growth performance and regulates lipid metabolism of juvenile Chinese mitten crab (*Eriocheir sinensis*) fed different percentage of lipid

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Abstract

This study evaluated the effects of dietary *myo*-inositol (MI) on growth performance, antioxidant status and lipid metabolism of juvenile Chinese mitten crab (*Eriocheir sinensis*) fed different percentage of lipid. Crabs (4·58 (SEM 0·05) g) were fed four diets including a normal lipid diet (N, containing 7 % lipid and 0 mg/kg MI), N with MI supplementation (N + MI, containing 7 % lipid and 1600 mg/kg MI), a high lipid diet (H, containing 13 % lipid and 0 mg/kg MI) and H with MI supplementation (H + MI, containing 13 % lipid and 1600 mg/kg MI) for 8 weeks. The H + MI group showed higher weight gain and specific growth rate than those in the H group. The dietary MI could improve the lipid accumulations in the whole body, hepatopancreas and muscle as a result of feeding on the high dietary lipid (13 %) in crabs. Besides, the crabs fed the H + MI diets increased the activities of antioxidant enzymes but reduced the malondialdehyde content in hepatopancreas compared with those fed the H diets. Moreover, dietary MI enhanced the expression of genes involved in lipid oxidation and exportation, yet reduced lipid absorption and synthesis genes expression in the hepatopancreas of crabs fed the H diet, which might be related to the activation of inositol 1,4,5-trisphosphate receptor (IP3R)/calmodulin-dependent protein kinase kinase-*β* (CaMKK*β*)/adenosine 5'-monophosphate-activated protein kinase (AMPK) signalling pathway. This study demonstrates that MI could increase lipid utilisation and reduce lipid deposition in the hepatopancreas of *E. sinensis* fed a high lipid diet through IP3R/CaMKK*β*/AMPK activation. This work provides new insights into the function of MI in the diet of crustaceans.

Key words: ***Myo*-inositol: *Eriocheir sinensis*: Lipid metabolism: Regulatory pathways: High lipid diet**

Lipids are critical to maintain the homoeostasis of cellular energy and serve various functions in animals, including aquatic animals. Lipids are also considered a lower-cost ingredient compared with protein, and high lipid diets have been used for a protein-sparing effect in aquaculture in recent years[1]. However, excess lipid content in diets can cause abnormal lipid deposition and oxidative stress, which would lead to metabolic disturbance and impair the growth, health and quality of aquatic animals[2,3]. Therefore, it is necessary to develop an appropriate strategy to enhance the utilisation of dietary lipid to promote growth, which benefits cost-effective aquaculture production. *Myo*-inositol (MI) is the most abundant isomeric form of inositol in living cells and feed ingredients. It has beneficial effects on varieties of diseases including polycystic ovary syndrome, type 2 diabetes and some other metabolic syndromes[4,5], making this isomer as one of the best candidates as a functional ingredient in a feed[6]. Moreover, the effect of MI as a major lipotropic factor has been well investigated in mammals[4,7–10]. A previous study reported that MI treatment could effectively reduce the white adipose tissue accretion of mice compared with saline solution treatment[11]. In addition, dietary MI could reduce the total lipid and TAG contents in the liver by depressing the hepatic activities of glucose-6-phosphate dehydrogenase, malic enzyme, fatty acid synthetase and citrate cleavage enzyme[12]. In aquatic

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, adenosine 5'-monophosphate-activated protein kinase; CaMKK*β*, calmodulin-dependent protein kinase kinase-*β*; CPT1, carnitine palmitoyltransferase I; IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; MI, *myo*-inositol; MDA, malondialdehyde; SOD, superoxide dismutase;; SREBP1, sterol regulatory element-binding protein 1; T-CHO, cholesterol.

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animals, previous studies have also demonstrated the function of MI in reducing lipid deposition[13–16]. For example, a negative correlation between dietary MI and hepatic lipid content exists in parrot fish (*Oplegnathus fasciatus*)[15]. In crustaceans, dietary MI is beneficial for reducing the midgut gland index and lowering lipid accumulation in the hepatopancreas of grass shrimp[16]. However, to our best knowledge, although studies have investigated the effects of dietary MI level on lipid deposition in fish, crustaceans and mammals, the underlying molecular mechanisms remain unclear and deserve further investigation.

The adenosine 5'-monophosphate-activated protein kinase (AMPK), as a cellular 'energy sensor', is an essential regulator of cellular fatty acid metabolism. The activation of AMPK could inhibit several sterol regulatory element-binding protein 1 (SREBP1) enzymes involved in fatty acid synthesis[17]. Moreover, the activation of AMPK can also phosphorylate acetyl-CoA carboxylase (ACC) leading to the decrease of malonyl-CoA, which could enhance lipid oxidation by regulating the expression of carnitine palmitoyltransferase I (CPT1)[18,19]. Besides, knockout of the AMPK gene could cause fatty liver and obesity in mammals[20]. Therefore, AMPK is considered an important target for regulating lipid metabolism in mammals[21]. Calmodulin-dependent protein kinase kinase-β (CaMKKβ), a member of the calmodulin kinase family[22], can phosphorylate AMPK at Thr^{172} in the catalytic α-subunit, and this process depends on the changes in intracellular Ca^{2+} concentration[23,24]. Besides, inositol 1,4,5-trisphosphate (IP3), the most important metabolite of inositol, can bind to the IP3 receptor (IP3R) on the endoplasmic reticulum, which could activate Ca^{2+} channels to release Ca^{2+} from the endoplasmic reticulum to the cytoplasm[25,26]. Previous studies reported that there was a close link between the regulation of AMPK activity and IP3R-mediated Ca^{2+} signalling pathway[27,28]. So far, many investigations have reported that MI plays a crucial part in the regulation of lipid metabolism in aquatic animals[13,15,29]. However, in contrast, no information has been reported on the specific molecular mechanism of MI regulating lipid metabolism.

The Chinese mitten crab (*Eriocheir sinensis*), one of the most important economic freshwater crustacean species, is a popular food crustacean in China and other Asian countries due to delicious meat, unique flavour and high protein. Previous studies in our laboratory showed that dietary 13 % lipid caused excessive lipid accumulation and oxidative stress in the hepatopancreas of *E. sinensis* compared with those fed 7–8 % lipid level[30,31]. This study aimed to use *E. sinensis* as a model species of crustacean to explore the influences of dietary MI on improvement of the negative effect of high lipid diet on the growth and antioxidant status in Chinese mitten crab. Particularly, lipid metabolism mediated by dietary MI was explored at different lipid levels to understand the role of MI in promotion of lipid utilisation in crustacean.

Materials and methods

Experimental diets

Four experimental diets (crude protein, approximately 44·0 %) were formulated with different lipid levels (7·0 and 13·0 %) and MI levels (0 and 1600 mg/kg diet) with four replicates. Casein and gelatine were used as the main protein sources, and fish oil, soyabean oil, lecithin and cholesterol were the lipid source. The formulation and proximate composition of four experimental diets are shown in Table 1. The diets were prepared with the process described in detail in our previous study[29]. The experimental diets were stored at –20°C until use.

Table 1. Ingredient formulation (g/kg dry basis) and proximate composition (%) of the four experimental diets fed to *Eriocheir sinensis*

	7 %		13 %	
	0	1600	0	1600
	N	N + MI	H	H + MI
Casein (vitamin-free)	360	360	360	360
Gelatin	90	90	90	90
Maize starch	250	250	250	250
Fish oil: soyabean oil (1:1)	60	60	120	120
Vitamin premix*	40	40	40	40
Mineral premix†	20	20	20	20
Choline chloride‡	5	5	5	5
Cholesterol‡	5	5	5	5
Betaine‡	30	30	30	30
Lecithin§	10	10	10	10
Cellulose	110	108·4	50	48·4
Carboxymethyl cellulose	20	20	20	20
Myo-inositol‡ (mg/kg diet)	0	1600	0	1600
Proximate composition (%)				
Moisture	9·11	9·02	9·39	9·25
Crude protein	44·05	44·05	43·75	44·25
Crude lipid	6·91	7·26	12·90	13·12
Ash	4·47	4·40	4·36	4·24

* Vitamin premix (per 100 g premix): retinol acetate, 0·043 g; thiamine hydrochloride, 0·15 g; riboflavin, 0·0625 g; Ca pantothenate, 0·3 g; niacin, 0·3 g; pyridoxine hydrochloride, 0·225 g; para-aminobenzoic acid, 0·1 g; ascorbic acid, 0·5 g; biotin, 0·005 g; folic acid, 0·025 g; cholecalciferol, 0·0075 g; α-tocopherol acetate, 0·5 g; menadione, 0·05 g. All ingredients are filled with α-cellulose to 100 g.

† Mineral premix (per 100 g premix): KH_2PO_4, 21·5 g; NaH_2PO_4, 10·0 g; $Ca(H_2PO_4)_2$, 26·5 g; $CaCO_3$, 10·5 g; KCl, 2·8 g; $MgSO_4{\cdot}7H_2O$, 10·0 g; $AlCl_3{\cdot}6H_2O$, 0·024 g; ZnSO4·$7H_2O$, 0·476 g; $MnSO_4$ $6H_2O$, 0·143 g; KI, 0·023 g; $CuCl_2{\cdot}2H_2O$, 0 ·015 g; $CoCl_2{\cdot}6H_2O$, 0 ·14 g; calcium lactate, 16·50 g; Fe-citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g.

‡ Sangong Biotech, Ltd.

§ Shanghai Taiwei, Ltd.

Crab rearing and experimental conditions

The feeding trial was conducted at Zhejiang Freshwater Fisheries Research Institute (Huzhou, China). Similar sized, active and intact crabs were obtained from a local farm (Shanghai, China) and fed a mixture of equal proportions of the experimental diets for 5 d to acclimatise to the environmental conditions. A total of 640 juvenile crabs (4·58 (SEM 0·05) g) were randomly distributed into sixteen tanks, with forty crabs per tank. Four arched tiles and four groups of corrugated plastic pipes were placed in each tank as shelters to reduce attacking behaviour. Crabs were fed three times daily (4 % of biomass) at 07.00, 16.30 and 22.30 for 8 weeks. After feeding, all tanks were cleaned daily by siphoning out the feed residue and faeces, and the water of 30 % tank volume was exchanged daily to maintain water quality. During the experiment, the water conditions were as follows: temperature 25 (SEM 2) °C, dissolved oxygen >7·0 mg/l, pH 8·0 (SEM 0·4) and ammonia and N <0·05 mg/l.

Sample collection and animal ethics

At the termination of the experiment, crabs were deprived of diet for 24 h, and counted and weighed to determine weight gain, specific growth rate, survival and feed conversion ratio. Four crabs per tank were collected and frozen (–20°C) until analysis of the proximate body composition. Haemolymph samples from five crabs per tank were collected immediately according to the method described by our previous study[32]. Haemolymph samples were placed into 1·5 ml Eppendorf tubes at 4°C for 24 h, followed by centrifugation at 4500 rpm for 10 min at 4°C. The supernatant was collected and stored at –80°C; until use. The hepatopancreas from three crabs in each tank was obtained to determine the hepatosomatic index, and hepatopancreas and muscle were quickly taken from these crabs, frozen in liquid N_2 and stored at –80°C for the analysis of proximate composition. Three crabs from each tank were sampled for analyses of biochemical parameters and enzyme activities in the hepatopancreas. The hepatopancreas tissues from three other crabs in each tank were collected, frozen in liquid N_2 and stored at –80°C to determine the expression of genes and proteins.

The study was conducted strictly according to the Guidance of the Care and Use of Laboratory Animals in China. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (No. f20201001).

Proximate composition analysis

Proximate analyses of diets, whole body, hepatopancreas and muscle were determined by the standard methods[33]. Ash was measured in a muffle furnace at 550°C for 6 h. Crude protein was measured by the Kjeldahl method (using Kjeltec™ 8200, Foss) and total lipid was measured by chloroform/methanol method[34]. Samples were dried to constant weight at 105°C to measure the moisture content. The MI content in hepatopancreas was measured by the enzymatic assay[35]. Briefly, the assay reaction was to convert MI into stable Iodonitrotetrazolium-formazan which was measured spectrophotometrically at 492 nm wavelength.

Biochemical analysis

Hepatopancreas samples were weighed and homogenised on ice in nine volumes (v/w) of ice-cold saline 8·9 g/ml, and then centrifuged at 2500 ***g*** at 4°C for 30 min. The supernatant was collected and stored at −80°C until analysis. The total antioxidant capacity (2, 2-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid diammonium salt) method, 405 nm), the activities of superoxide dismutase (SOD, hydroxylamine method, 550 nm) and glutathione peroxidase (5, 50-dithiobis-(2-nitrobenzoic acid) method, 412 nm), malondialdehyde (MDA, thiobarbituric acid method, 532 nm) content and TAG (glycerophosphate oxidase – peroxidase method, 510 nm), cholesterol (T-CHO, cholesterol oxidase - peroxidase) method, 510 nm), NEFA (acetyl CoA synthase - acetyl CoA oxidase - peroxidase method, 546 nm) and total protein content (bicinchoninic acid method, 562 nm) in hepatopancreas were measured using the diagnostic reagent kits (Cat. No. A015-2, A001-3, A005-1, A003-1, A110-1, A111-1, A042-2 and A045-4, Nanjing Jiancheng Bioengineering Institute).

The TAG (glycerophosphate oxidase – peroxidase method, 510 nm), T-CHO (cholesterol oxidase - peroxidase method, 510 nm), NEFA (acetyl CoA synthase - acetyl CoA oxidase - peroxidase method, 546 nm), LDL-cholesterol (enzymatic method, 546 nm) and HDL-cholesterol (enzymatic method, 546 nm) contents in haemolymph were measured using the diagnostic reagent kits (Cat. No. A110-1, A111-1, A042-2, A113-1, A112-1, Nanjing Jiancheng Bioengineering Institute).

RNA extraction and quantitative real-time PCR analysis

The total RNA was isolated from hepatopancreas using RNAiso Plus (Takara) and was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent kit (Takara). The integrity and quality of RNA were determined by agarose gel electrophoresis and spectrophotometry. The quantitative real-time (RT)-PCR was carried out in the CFX96 Real-Time PCR system (Bio-rad, Richmond) and conducted in a final reaction volume of 20 μl containing 10 μl of SYBR qPCR reagent (Q711-02/03, Vazyme Biotech Co., Ltd), 0·4 μl (10 μM) of each forward and reverse primers, 8·2 μl of DEPC-H_2O and 1 μl cDNA template. The programme of RT-PCR reactions was the same as in our previous study[29]. The amplification efficiency of genes was analysed according to the eq. $E = 10^{(-1/Slope)} - 1$ and the E-values ranged from 90·1 to 102·6 %. The β-actin and S27 genes were used as an internal control, and the quantitative RT-PCR was quantified by the $2^{-\Delta\Delta Ct}$ method[36]. The primers used are shown in Table 2.

Western blotting

Immunoblot analyses were performed according to the procedure in our laboratory[37]. Briefly, protein homogenates were prepared from liver tissues in the cell lysis buffer (Beyotime Biotechnology) containing 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology). After 10 400 ***g*** centrifugation for 10 min, the supernatant was mixed with 5 × SDS loading buffer and boiled at 95°C for 5 min. Protein concentrations were determined using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo, Scientific). Proteins (20–50 μg) were separated on SDS-PAGE and transferred electrophoretically to nitrocellulose filter membranes. The membranes were blocked with 5 % non-fat milk in tris-buffered saline (TBS) with 0.05% Tween 20 and were incubated overnight at 4°C with primary antibodies against phospho-IP3R (DF2999, Affinity), phospho-AMPKα (AF3423, Affinity), phospho-ACC1 (AF3421, Affinity) and β-actin (AB0061, Abways). After washing, membranes were incubated with anti-rabbit or mouse horseradish peroxidase-conjugated secondary antibody. The images of Western blotting were determined using the Odyssey CLx Imager (Licor), and the target proteins were quantified using the ImageJ 1.44p software (US National Institutes of Health).

Calculation and statistical analysis

The calculation formulas can be found in online Supplementary File. Results are presented as mean values with their standard error of the mean. Data were checked for normality and

Table 2. Primer pair sequences and product size of the genes used for real-time PCR (qPCR)

Gene	Position	Primer sequence	Length	Tm	Product size (bp)
Lipogenesis-related genes					
srebp 1	Forward	TCTTCACACCCTCTGGACGC	20	60·5	162
	Reverse	CCAAGGTTGTAATGGCACGC	20	61·3	
fas	Forward	GTCCCTTCTTCTACGCCATCC	21	60·3	127
	Reverse	CGCTCTCCAGGTCAATCTTCAC	22	61·3	
dgat 1	Forward	CGAGCACATAACACAAGCGG	20	57·5	98
	Reverse	AACCAGACGACCACGAGAAC	20	57·5	
Δ9 fad	Forward	TGGCACAACTACCACCACGTCT	22	63·1	160
	Reverse	TCCTCTTCTCGATCATCTCCGG	22	62·8	
Lipid catabolism-related genes					
cpt 1a	Forward	CATCTGGACACCCACCTCCA	20	60·8	183
	Reverse	ATCTCCTCACCCGGCACTCT	20	60·7	
cpt 1b	Forward	GGCATTCTCCTTTGCCATCAC	21	61·8	138
	Reverse	ACACCACACCGCACATTGTTC	21	61·2	
cpt 2	Forward	AGCAGGCAGTGGCTCAGTTTA	21	60·2	169
	Reverse	AAGGCAAGGAAGGGGTTGTAG	21	60·1	
Fatty acid uptake-related genes					
fabp 3	Forward	CCACCGAGGTCAAGTTCAAGC	21	58·4	195
	Reverse	TCACACCATCACACTCCGACAC	22	59·5	
fabp 9	Forward	GCCGCACCTCAACTCCACTACAA	23	66·1	108
	Reverse	ATCACCAGTCCCACACCCAAAGC	23	66·9	
fabp 10	Forward	TGCTGATTGGCTCAGTGCTGTG	22	64·4	115
	Reverse	CGTGGTCTTGATGACGATGTCG	22	63·5	
Lipid transport-related gene					
mttp	Forward	TAGGACAAGCAGGACTTTCCTCA	23	60·8	138
	Reverse	CCACATCCACAAACACATCAACA	23	61·3	
AMPK pathway-related gene					
camkkβ	Forward	CCATCCTGAAGAAGCTGAAC	20	55·4	96
	Reverse	GGAGCTCAAACACCATGTAG	20	55·4	
Reference genes					
β-actin	Forward	TCGTGCGAGACATCAAGGAAA	21	61·5	178
	Reverse	AGGAAGGAAGGCTGGAAGAGTG	22	61·6	
S27	Forward	CCCCCAAGAAGATCAAGCACA	21	62·3	179
	Reverse	CAGATGGCAGCGACCACAGTA	21	61·8	

srebp1, sterol regulatory element-binding protein 1; *fas*, fatty acid synthase; *dgat1*, diacylglycerol O-acyltransferase 1; *Δ9 fad*, Δ9 fatty acyl desaturase; *cpt*, carnitine palmitoyltransferase; *fabp*, fatty acid binding protein; *mttp*, microsomal TAG transfer protein; *camkkβ*, calmodulin-dependent protein kinase kinase-*β*; *S27*, ubiquitin/ribosomal S27 fusion protein.

homogeneity of variances, and they were normalised when appropriate. Data were analysed by two-way ANOVA to determine if there was any interaction between dietary lipid level and MI level. At the same dietary lipid level, independent-samples *t* test was used to analyse the significant differences between crabs fed diets with 0 and 1600 mg/kg MI. At the same dietary MI level, independent-samples *t* test was used to determine significant differences between crabs fed diets with 7 and 13 % lipid. The level of significance was set at $P < 0·05$. All statistical analyses were conducted using the SPSS 20.0 software package for Windows (SPSS).

Result

Growth performance, feed utilisation and hepatosomatic index

The weight gain and specific growth rate were significantly influenced by MI level ($P < 0·05$, online Supplementary Table S1). The H + MI group (13 % lipid and 1600 mg/kg MI) showed significantly higher weight gain (Fig. 1(a)) and specific growth rate (Fig. 1(b)) than the H group (13 % lipid and 0 mg/kg MI, $P < 0·05$). Dietary MI supplementation also had a positive impact on weight gain (Fig. 1(a)) and specific growth rate (Fig. 1(b)) when the lipid level was 7 %, though there were no significant differences between the N (7 % lipid and 0 mg/kg MI) and N + MI groups (7 % lipid and 1600 mg/kg MI, $P > 0·05$). There was no significant difference in survival among the groups ($P > 0·05$) (Fig. 1(c)). Crabs fed the N (7 % lipid and 0 mg/kg MI) diet had a markedly lower FCR than those fed the H (13 % lipid and 0 mg/kg MI) diet ($P < 0·05$) (Fig. 1(d)). The hepatosomatic index declined in N + MI (7 % lipid and 1600 mg/kg MI) and H + MI (13 % lipid and 1600 mg/kg MI) groups when compared with the N (7 % lipid and 0 mg/kg MI) and H (13 % lipid and 0 mg/kg MI) groups, respectively ($P < 0·05$) (Fig. 1(e)).

Proximate compositions of tissues and myo-inositol content in hepatopancreas

The whole-body lipid content was markedly affected by dietary MI level ($P < 0·05$), and the total lipid content in hepatopancreas and muscle, whole-body protein content and hepatopancreas MI content were markedly affected by dietary lipid and MI levels ($P < 0·05$, online Supplementary Table S2). No significant difference was observed in whole-body moisture content among the groups ($P > 0·05$) (Fig. 2(a)). Crabs fed 13 % lipid showed a significantly lower crude protein level in the whole body than those fed 7 % lipid regardless of dietary MI ($P < 0·05$) (Fig. 2(b)).

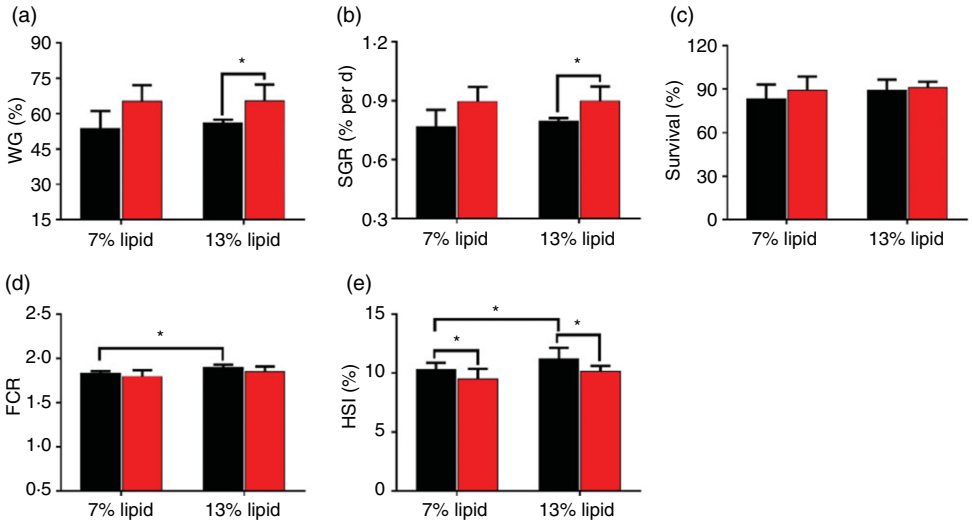

Fig. 1. Effects of dietary *myo*-inositol on growth performance, feed utilisation and HSI of *Eriocheir sinensis* fed different lipid level. Values are means (n 4 replicate tanks) with standard errors represented by vertical bars. * indicates significant difference between *myo*-inositol levels within the same lipid level or between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$). WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; HSI, hepatosomatic index. ■, 0 mg/kg; ■, 1600 mg/kg.

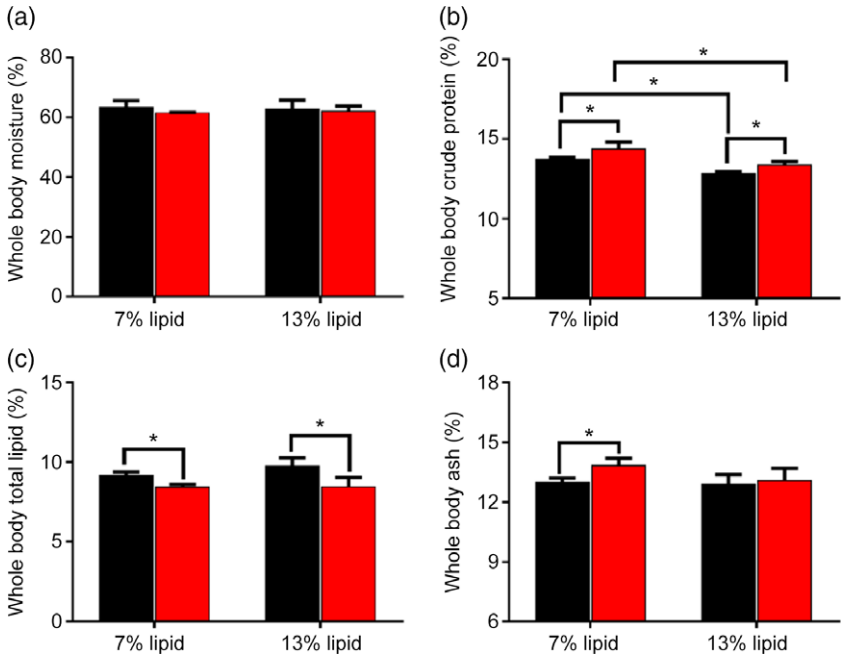

Fig. 2. Effects of dietary *myo*-inositol on the whole-body proximate compositions of *Eriocheir sinensis* fed different lipid level. Values are means (n 4 replicate tanks) with standard errors represented by vertical bars. * indicates significant difference between *myo*-inositol levels within the same lipid level or between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$). ■, 0 mg/kg; ■, 1600 mg/kg.

Moreover, the whole-body protein content significantly increased in the N + MI (7 % lipid and 1600 mg/kg) and H + MI (13 % lipid and 1600 mg/kg MI) groups when compared with the N (7 % lipid and 0 mg/kg MI) and the H (13 % lipid and 0 mg/kg MI) groups, respectively ($P < 0{\cdot}05$) (Fig. 2(b)). The whole-body lipid content decreased in N + MI (7 % lipid and 1600 mg/kg MI) and H + MI (13 % lipid and 1600 mg/kg MI) groups when compared with the N (7 % lipid and 0 mg/kg MI) and H (13 % lipid and 0 mg/kg MI) groups, respectively ($P < 0{\cdot}05$) (Fig. 2(c)). Dietary MI supplementation markedly increased whole-body ash content when the lipid level was 7 % ($P < 0{\cdot}05$) (Fig. 2(d)). Crabs fed 13 % lipid showed a significantly lower crude protein level in the hepatopancreas than those fed 7 % lipid regardless of dietary MI ($P < 0{\cdot}05$) (Fig. 3(a)). No significant difference was observed in the crude protein level in muscle among the groups ($P > 0{\cdot}05$) (Fig. 3(b)). Total lipid contents in hepatopancreas (Fig. 3(c)) and muscle (Fig. 3(d)) in the H (13 % lipid and 0 mg/kg MI) group were markedly higher than those in the N group (7 % lipid and 0 mg/kg MI, $P < 0{\cdot}05$). However, total lipid contents in

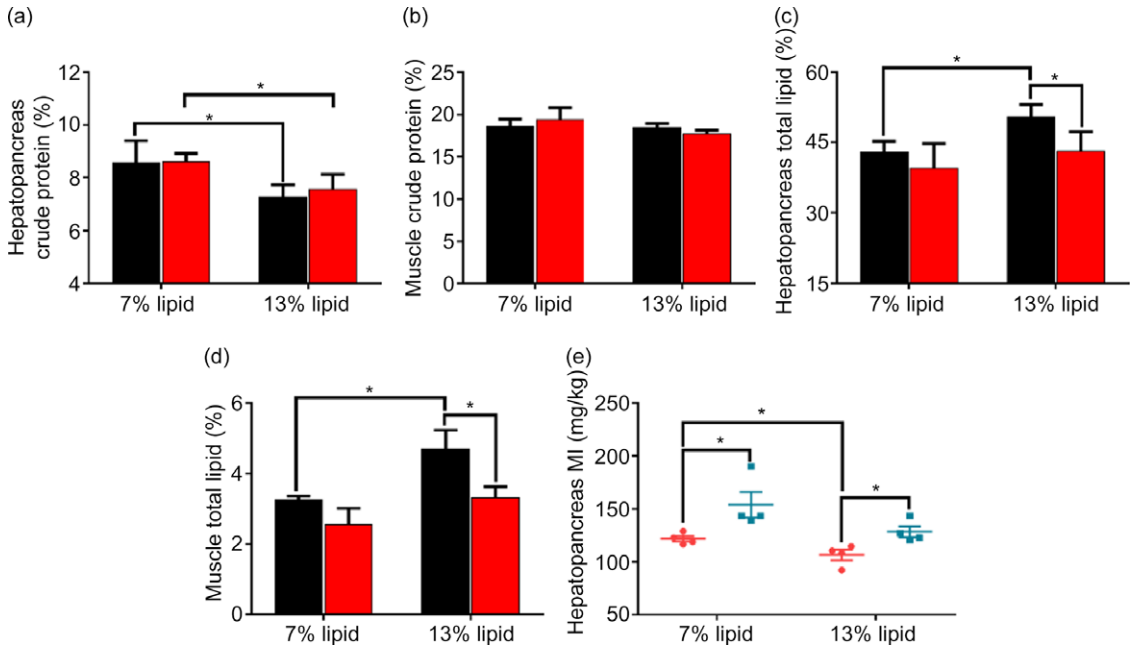

Fig. 3. Effects of dietary *myo*-inositol on proximate compositions of the hepatopancreas and muscle of *Eriocheir sinensis* fed different lipid level. Values are means (n 4 replicate tanks) with standard errors represented by vertical bars. * indicates significant difference between *myo*-inositol levels within the same lipid level or between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$). MI, *myo*-inositol. ■, 0 mg/kg; ■, 1600 mg/kg.

hepatopancreas (Fig. 3(c)) and muscle (Fig. 3(d)) were significantly reduced in the H + MI (13 % lipid and 1600 mg/kg MI) group compared with those in the H (13 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$). Crabs fed the H (13 % lipid and 0 mg/kg MI) diet had a markedly lower MI level in hepatopancreas than those fed the N (7 % lipid and 0 mg/kg MI) diet ($P < 0{\cdot}05$) (Fig. 3(e)). Dietary MI supplementation markedly increased MI level in hepatopancreas regardless of dietary lipid ($P < 0{\cdot}05$) (Fig. 3(e)).

Biochemical indicators in haemolymph and hepatopancreas

As shown in Table 3, there were significant main effects of dietary MI on the TAG, T-CHO and NEFA contents in hepatopancreas and T-CHO and LDL-cholesterol contents in haemolymph ($P < 0{\cdot}05$). The TAG and T-CHO contents were also affected by the interaction between dietary MI and lipid levels ($P < 0{\cdot}05$). Crabs in the H (13 % lipid and 0 mg/kg MI) group had significantly higher TAG, T-CHO and NEFA levels in haemolymph than those in the N (7 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$). Dietary 1600 mg/kg MI significantly reduced the TAG, T-CHO and LDL-cholesterol levels in haemolymph compared with those fed diets without MI supplementation when the lipid level was 13 % ($P < 0{\cdot}05$). No significant difference was observed in the HDL-cholesterol level in haemolymph among the groups ($P > 0{\cdot}05$). Crabs fed 1600 mg/kg MI showed a significantly lower TAG content in hepatopancreas than those fed 0 mg/kg MI regardless of dietary lipid ($P < 0{\cdot}05$). The T-CHO and NEFA contents of the hepatopancreas in the H + MI (13 % lipid and 1600 mg/kg MI) group were significantly lower than those in the H (13 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$).

Antioxidant indicators in hepatopancreas

The SOD activity and MDA content were significantly affected by dietary MI and the interaction between MI and lipid ($P < 0{\cdot}05$). Crabs in the H (13 % lipid and 0 mg/kg MI) group had significantly lower total antioxidant capacity and the activities of SOD and glutathione peroxidase, and a higher MDA content in hepatopancreas than those in the N (7 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$). The increased total antioxidant capacity and the activities of SOD and glutathione peroxidase, and the decreased MDA content in hepatopancreas were found in the H + MI (13 % lipid and 1600 mg/kg MI) group compared with those in the H (13 % lipid and 0 mg/kg MI) group (Table 3, $P < 0{\cdot}05$).

Gene expression

As shown in Fig. 4 and online Supplementary Table S3, the mRNA abundances of *srebp1*, *dgat1*, *cpt2*, *camkkβ*, *fabp10* and *mttp* were markedly influenced by dietary lipid and MI ($P < 0{\cdot}05$), and the mRNA abundances of *fas*, *Δ9 fad* and *fabp3* were dramatically influenced by dietary lipid, MI and their interaction ($P < 0{\cdot}05$). Specifically, the H (13 % lipid and 0 mg/kg MI) group showed a markedly higher relative expression of genes involved in lipid synthesis (*srebp1*, *fas* and *Δ9* fad) than the N (7 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$), but no significant difference was found in *dgat1* between the two groups ($P > 0{\cdot}05$). Crabs fed 1600 mg/kg MI showed significantly lower mRNA abundance of *srebp1*, *fas* and *Δ9* fad than those fed 0 mg/kg MI regardless of dietary lipid ($P < 0{\cdot}05$). Moreover, crabs in the H + MI (13 % lipid and 1600 mg/kg MI) group had a significantly lower mRNA abundances of *dgat1* than those in the H (13 % lipid and 0 mg/kg MI) group ($P < 0{\cdot}05$). The H + MI (13 % lipid and 1600 mg/kg MI) group

Table 3. Biochemical indicators in haemolymph and hepatopancreas and antioxidant indicators in hepatopancreas of *Eriocheir sinensis* fed different diets* (Mean values with their standard error of the mean, *n* 4)

	7 %				13 %						
	0 mg/kg		1600 mg/kg		0 mg/kg		1600 mg/kg				
Lipid	N		N + MI		H		H + MI		Two-way ANOVA (*P*)		
Myo-inositol	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	LL	ML	LL × ML
Biochemical indicators in haemolymph											
TAG (mmol/l)	$0{\cdot}09^{B}$	0·01	$0{\cdot}09^{B}$	0·00	$0{\cdot}17^{aA}$	0·01	$0{\cdot}14^{bA}$	0·01	<0·01	NS	<0·05
T-CHO (mmol/l)	$0{\cdot}77^{B}$	0·04	$0{\cdot}79^{B}$	0·03	$1{\cdot}35^{aA}$	0·02	$1{\cdot}07^{bA}$	0·05	<0·01	<0·01	<0·01
HDL-cholesterol (mmol/l)	0·12	0·01	0·12	0·02	0·09	0·01	0·11	0·01	NS	NS	NS
LDL-cholesterol (mmol/l)	0·11	0·01	0·09	0·01	0·16a	0·01	$0{\cdot}09^{b}$	0·01	NS	<0·01	NS
NEFA (mmol/l)	$0{\cdot}06^{B}$	0·01	0·07	0·01	$0{\cdot}09^{A}$	0·01	0·09	0·01	<0·01	NS	NS
Biochemical indicators in hepatopancreas											
TAG (mmol/gprot)	$9{\cdot}64^{aB}$	0·33	$6{\cdot}52^{bB}$	0·77	$11{\cdot}86^{aA}$	0·72	$9{\cdot}42^{bA}$	0·46	<0·01	<0·01	NS
T-CHO (mmol/gprot)	0·10	0·01	0·08	0·01	0·13a	0·02	$0{\cdot}07^{b}$	0·01	NS	<0·01	NS
NEFA (mmol/gprot)	0·06	0·01	0·03	0·00	0·09a	0·02	$0{\cdot}04^{b}$	0·01	NS	<0·01	NS
Antioxidant indicators in hepatopancreas											
SOD (U/mgprot)	$8{\cdot}40^{A}$	0·58	8·73	0·58	$4{\cdot}76^{bB}$	0·04	$10{\cdot}48^{a}$	1·56	NS	<0·01	<0·05
T-AOC (mmol/gprot)	$0{\cdot}16^{bA}$	0·00	$0{\cdot}21^{a}$	0·01	$0{\cdot}13^{bB}$	0·01	$0{\cdot}23^{a}$	0·03	NS	<0·01	NS
GSH-Px (U/mgprot)	$156{\cdot}36^{bA}$	4·95	$188{\cdot}64^{a}$	11·63	$125{\cdot}57^{bB}$	7·51	$175{\cdot}19^{a}$	13·50	<0·05	<0·01	NS
MDA (nmol/mgprot)	$4{\cdot}43^{aB}$	0·23	$3{\cdot}16^{b}$	0·36	$6{\cdot}50^{aA}$	0·59	$3{\cdot}15^{b}$	0·47	<0·05	<0·01	<0·05

T-CHO, cholesterol; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; NS, no significant difference; LL, lipid levels; ML, *myo*-inositol levels; LL × ML, lipid levels × *myo*-inositol levels.
* Values in the same row with different superscripts are significantly different ($P < 0{\cdot}05$).
^{A,B}Means significant difference between *myo*-inositol levels within the same lipid level ($P < 0{\cdot}05$).
^{a,b}Means significant difference between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$).

showed significantly higher transcriptional levels of *cpt1a*, *cpt1b* and *cpt2* than the H group ($P < 0{\cdot}05$). Crabs fed 13 % lipid had a significantly higher transcriptional level of *cpt2* than those fed 7 % lipid regardless of dietary MI ($P < 0{\cdot}05$). In addition, crabs fed 1600 mg/kg MI had a markedly higher *camkkβ* mRNA level than those fed 0 mg/kg MI regardless of dietary lipid ($P < 0{\cdot}05$). The mRNA abundances of genes involved in fatty acid uptake (*fabp3* and *fabp10*) in the H group were significantly higher than those in the N group ($P < 0{\cdot}05$), but crabs fed the H + MI (13 % lipid and 1600 mg/kg MI) diet significantly down-regulated the transcriptional levels of these genes compared with those fed the H (13 % lipid and 0 mg/kg MI) diet ($P < 0{\cdot}05$). No significant difference was observed in the transcriptional level of *fabp9* among the groups ($P > 0{\cdot}05$). Crabs in the H (13 % lipid and 0 mg/kg MI) and H + MI (13 % lipid and 1600 mg/kg MI) groups had a significantly higher transcriptional level of *mttp* than those in the N (7 % lipid and 0 mg/kg MI) and N + MI (7 % lipid and 1600 mg/kg MI) groups, respectively ($P < 0{\cdot}05$). Furthermore, crabs fed 1600 mg/kg MI had a significantly higher transcriptional level of *mttp* than those fed 0 mg/kg MI regardless of dietary lipid ($P < 0{\cdot}05$).

Protein expression

There was a significant main effect of dietary MI on the p-IP3R, p-AMPK and p-ACC1 protein levels in hepatopancreas (online Supplementary Table S4, $P < 0{\cdot}05$). The p-IP3R (Fig. 5(a)), p-AMPK (Fig. 5(b)) and p-ACC1 (Fig. 5(c)) protein levels in crabs fed the N + MI (7 % lipid and 1600 mg/kg MI) and H + MI (13 % lipid and 1600 mg/kg MI) diets were significantly increased compared with those fed the N (7 % lipid and 0 mg/kg MI) and H (13 % lipid and 0 mg/kg MI) diets, respectively ($P < 0{\cdot}05$).

Discussion

In the present study, no significant differences were found in the survival and growth performance of Chinese mitten crab between the N and H groups. This result is consistent with the results of a previous study[30], which demonstrated that high lipid diets did not significantly impair the growth of crustaceans. However, excessive energy in diets may affect energy metabolism, leading to lipid accumulation in tissues, which further impairs organelle integrity by increasing the production of reactive oxygen species and the risk of oxidative stress in aquatic animals and mammals[38–40]. The MDA, a stable end product of fatty acid peroxidation, is a biomarker of oxidative stress[41], and the elevation of antioxidant enzymes, such as SOD and glutathione peroxidase, is one of the main approaches against the toxicity of reactive oxygen species[42]. Indeed, although the growth performance of crab was not markedly affected by dietary 13 % lipid, oxidative stress was induced by excess lipid deposition through increasing the risk of lipid peroxidation and disturbing the enzymatic–antioxidant system of *E. sinensis*, which is in line with the results in the previous studies on the same species[31] and other aquatic animals[43,44]. In contrast, dietary MI supplementation enhanced the growth performance and antioxidant capacity of *E. sinensis* under a high lipid diet, which is supported by the findings in other studies on fish and crustacean[15,16,29,45]. This result seemingly suggests that MI, as a cyclitol, can donate hydrogen and have a chelating character to reduce oxidative stress by scavenging hydroxyl radicals[46]. Similarly, dietary MI could prevent lipid peroxidation and protein oxidation induced by waterborne copper in muscle via NF-E2-related factor 2/antioxidant response element signalling pathway in Jian carp (*Cyprinus*

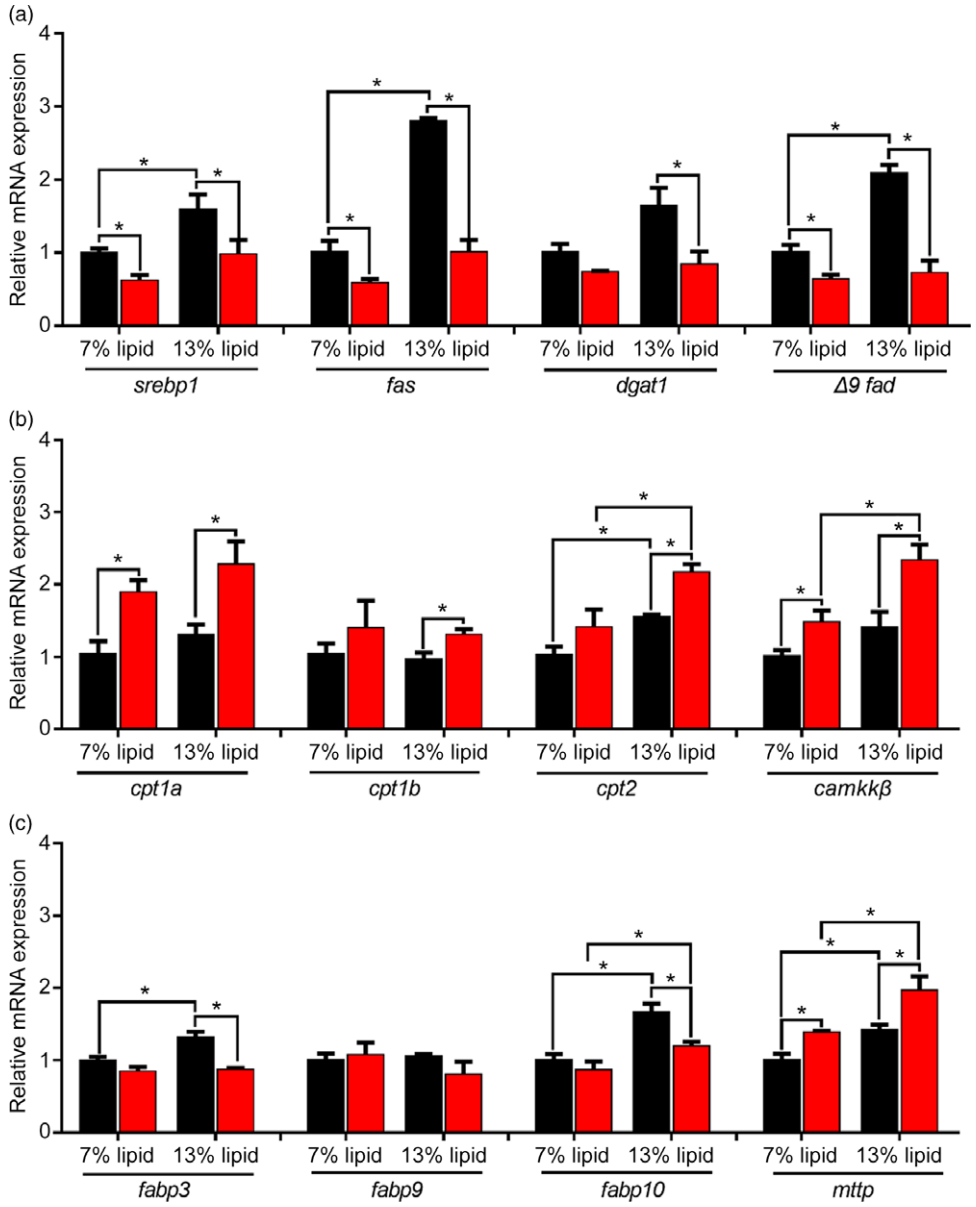

Fig. 4. Expression of genes involved in lipid metabolism in hepatopancreas of *Eriocheir sinensis* fed different experimental diets. Values are means (*n* 4 replicate tanks) with standard errors represented by vertical bars. * indicates significant difference between *myo*-inositol levels within the same lipid level or between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$). *srebp1*, sterol regulatory element-binding protein 1; *fas*, fatty acid synthase; *dgat1*, diacylglycerol O-acyltransferase 1; *Δ9 fad*, Δ9 fatty acyl desaturase; *cpt*, carnitine palmitoyltransferase; *camkkβ*, calmodulin-dependent protein kinase kinase-*β*; *fabp*, fatty acid binding protein; *mttp*, microsomal TAG transfer protein. ■, 0 mg/kg; ■, 1600 mg/kg.

carpio var. Jian)[47]. Collectively, dietary MI could reverse the lipid peroxidation and down-regulation of antioxidation enzyme activities caused by dietary high lipid and improve the health of *E. sinensis*. On the other hand, dietary MI could facilitate lipid utilisation in animals fed high lipid diet by optimising lipid metabolism, leading to fast growth[4,15,29].

The role of lipid reduction by MI has been reported in human, mammals and aquatic animals. Extra dietary MI can be beneficial in adiposity, hyperglycaemia and insulin resistance for human[4]. Dietary MI supplementation could reduce TAG accumulation in high fructose-induced fatty liver in rats[7]. In contrast, dietary MI deficiency could result in lipid accumulation in the liver of rats[48], fish[13–15] and crustaceans[16,29]. Furthermore, the increase of dietary lipid levels may result in the increase of body lipid and the decrease of body protein in aquatic animals[1,49]. The present study found that 13 % dietary lipid increased the hepatosomatic index and lipid contents in hepatopancreas and muscle of crabs compared with 7 % dietary lipid. This result is consistent with the results of the same species, which illustrates a positive relationship between dietary lipid and body lipid[30,31]. Moreover, dietary MI could reverse the reduction of protein in the whole body and lipid deposition in the whole body, hepatopancreas and muscle of *E. sinensis* fed the high (13 %) lipid diet, which is

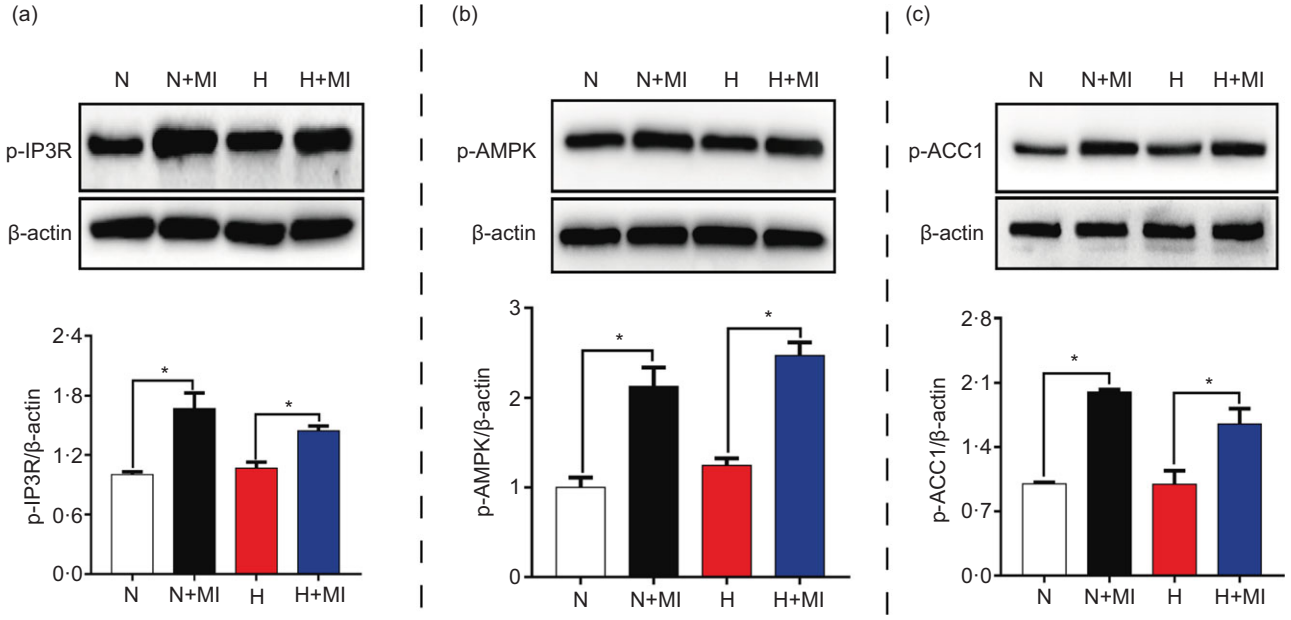

Fig. 5. Effects of dietary *myo*-inositol on protein levels of p-IP3R, p-AMPK and p-ACC1 of *Eriocheir sinensis* fed different lipid level. Values are means (n 4 replicate tanks) with standard errors represented by vertical bars. * indicates significant difference between *myo*-inositol levels within the same lipid level or between lipid levels within the same *myo*-inositol level ($P < 0{\cdot}05$). p-IP3R, phosphorylation of inositol 1,4,5-trisphosphate receptor; p-AMPK, phosphorylation of adenosine 5'-monophosphate-activated protein kinase; p-ACC1, phosphorylation of acetyl-CoA carboxylase 1.

similar to the results of a previous study[50]. These results indicate that MI may help achieve the protein-sparing effect in crab feed. Intriguingly, 13 % dietary lipid reduced the MI content in the hepatopancreas compared with 7 % dietary lipid, but dietary MI supplementation markedly enhanced the MI content in the hepatopancreas of crabs. This finding further reflects that the reduction of MI in hepatopancreas may be used to resist the negative effects of excessive lipid deposition induced by high lipid diet[6]. In other words, MI may play a vital role in reducing lipid accumulation of crabs and improving lipid utilisation.

In the present study, crabs fed the high (13 %) lipid diet showed increased TAG and cholesterol contents in haemolymph and TAG content in hepatopancreas when MI was not added to the diet, indicating an active endogenous lipid transport. This result is in agreement with some other reports on crustaceans and fish[40,44,51,52]. On the contrary, the reduced TAG, cholesterol and LDL-cholesterol in haemolymph and low TAG, cholesterol and NEFA in the hepatopancreas of crabs fed the high lipid diet with MI supplementation suggest that MI may accelerate the overall process of endogenous lipid consumption. In other words, MI was involved in lipid catabolism to supply the energy need of crabs for rapid growth. In addition, LDL carries cholesterol from the liver to peripheral tissues, while HDL carries cholesterol from peripheral tissues to the liver[53]. Therefore, the reduced cholesterol in the haemolymph of crabs fed the high lipid diet with MI supplementation is possibly attributed to the decreased LDL-cholesterol[54].

So far, the specific molecular mechanism of MI regulating lipid metabolism remains unclear in crustacean. The present study found that the mRNA abundances of genes involved in lipid uptake were up-regulated in the H group compared with the N group, suggesting that high dietary lipid could enhance lipid absorption in the hepatopancreas, resulting in an elevation of lipid accumulation in the hepatopancreas of *E. sinensis*, which is supported by the previous studies[31,55]. The present study also demonstrated that dietary MI effectively reversed the up-regulation of gene expressions in lipid uptake induced by high dietary lipid, resulting in slow lipid deposition in the hepatopancreas of crabs. Moreover, *mttp* is vital in the formation of VLDL in aquatic animals and mammals[56,57], and VLDL is responsible for transporting TAG from the hepatopancreas to perihepatic tissues[58]. Thus, the present result indicates that dietary MI might promote the secretion of VLDL to avoid excessive lipid accumulation. In addition to lipid uptake and transport, lipogenesis and lipid catabolism were also affected by dietary lipid and MI. The *Srebp1*, as a transcription factor, plays a crucial role in lipogenesis, which can activate downstream genes in different steps of fatty acid synthesis *de novo* and TAG synthesis including *fas*, *dgat1* and *Δ9 fad*[59–61]. In the present study, dietary MI decreased the expression of genes involved in fatty acid synthesis *de novo* for which the expression was increased by high dietary lipid, suggesting that dietary MI might reduce the lipid deposition in the hepatopancreas of crabs by down-regulating the transcriptional level of *srebp1* under high lipid diet. This result is similar to the results on mammals, where dietary supplementation with MI could reduce the expression of lipogenic genes and TAG accumulation in the high fructose-induced fatty liver in rats[7,62]. On the other hand, *β*-oxidation is a vital approach to lipid catabolism in crabs, fish and mammals[31,63,64]. CPT1 is involved in long-chain fatty acid oxidation, catalysing the conversion of fatty acid CoA to fatty acid carnitines for accessing the mitochondrial matrix, and the fatty acyl group is transferred back to CoA by CPT2[65]. The present study showed that dietary MI markedly up-regulated the mRNA abundances of *cpt1a*, *cpt1b* and *cpt2* in the hepatopancreas of crabs under

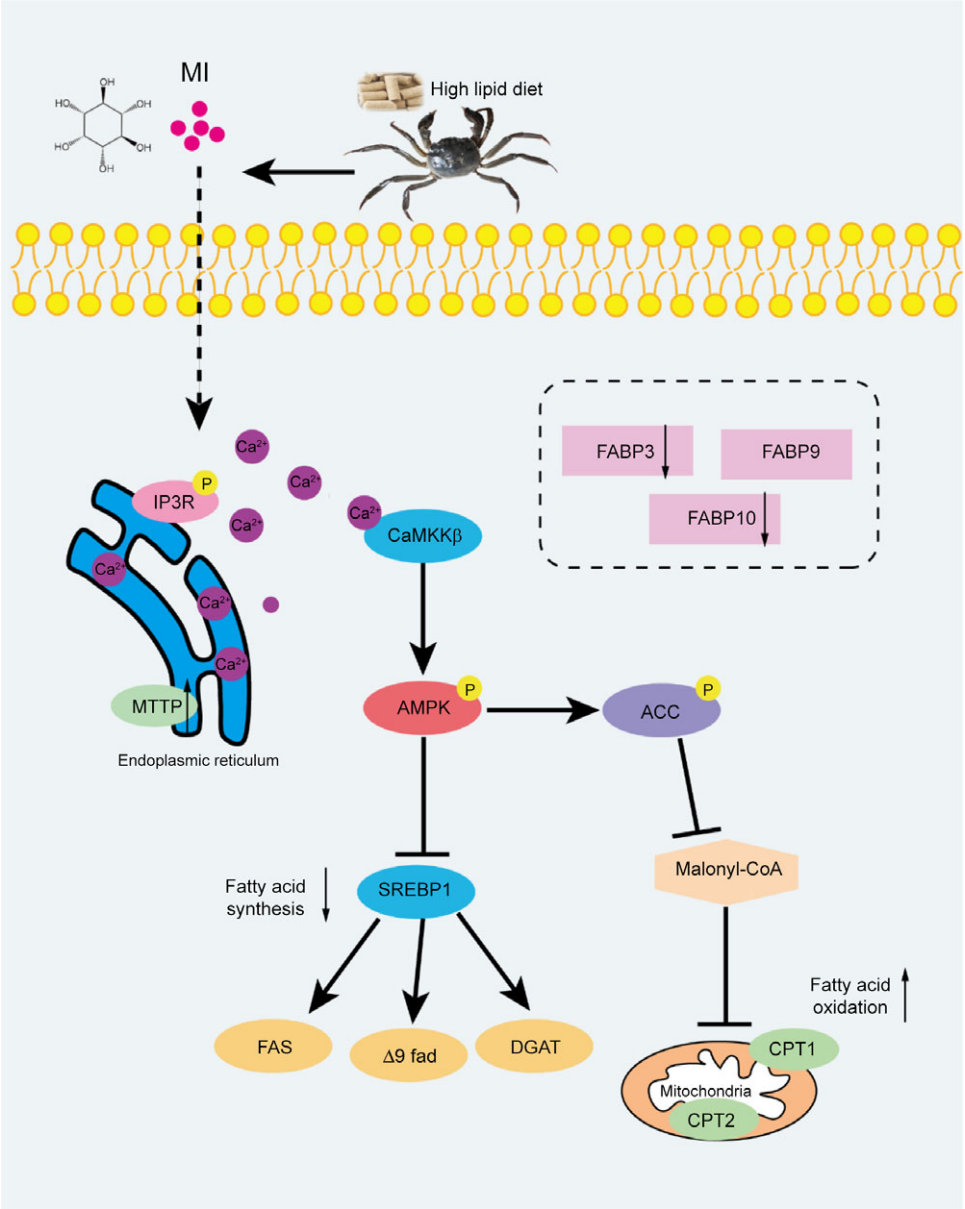

Fig. 6. The model of *myo*-inositol induced changes of fatty acid synthesis and oxidation via the IP3R/CaMKK*β*/AMPK pathway. SREBP1, sterol regulatory element-binding protein 1; FAS, fatty acid synthase; DGAT, diacylglycerol O-acyltransferase; Δ9 fad, Δ9 fatty acyl desaturase; CPT, carnitine palmitoyltransferase; CaMKK*β*, calmodulin-dependent protein kinase kinase-*β*; FABP, fatty acid binding protein; MTTP, microsomal TAG transfer protein; IP3R, inositol 1,4,5-trisphosphate receptor; AMPK, adenosine 5'-monophosphate-activated protein kinase; ACC, acetyl-CoA carboxylase.

high lipid diets, indicating the enhanced activity of fatty acid *β*-oxidation, which is consistent with the change of lipid content in the hepatopancreas of crabs. Therefore, dietary MI could lessen the lipid accumulation in the hepatopancreas of crabs induced by high lipid diet through inhibiting lipid absorption and synthesis and facilitating fatty acid *β*-oxidation.

AMPK can regulate lipid synthesis and decomposition[17]. For instance, dietary Zn can inhibit SREBP1-mediated fatty acid synthesis and promote fatty acid *β*-oxidation through the Ca^{2+}/CaMKK*β*/AMPK pathway[66]. Meanwhile, AMPK can phosphorylate and inactivate ACC, resulting in the reduction of malonyl-CoA, which is an inhibitor of CPT1[67]. The present results exhibit that dietary MI can inhibit the fatty acid synthesis and promote fatty acid entry into mitochondria for *β*-oxidation in the hepatopancreas of crab through AMPK activation. In addition, the present study shows that the IP3R was activated and *camkkβ* was up-regulated by dietary MI supplementation. A previous study reported that the activation of IP3R could cause an elevation of cytosolic Ca^{2+} and up-regulate CaMKK*β* with subsequent activation of its downstream molecule AMPK[28]. Moreover, IP3, as a second messenger, is the most crucial metabolite of inositol produced through phosphoinositide turnover and can bind to the IP3R on the endoplasmic reticulum to regulate cytosolic Ca^{2+} homoeostasis[26]. Thus, according to the metabolic fate of MI, MI may regulate lipid metabolism of *E. sinensis* through activating the IP3R/CaMKK*β*/AMPK

pathway. It is worth noting that the Ca^{2+} pathway-mediated AMPK activity regulated by IP3R is still not completely clear. For example, some studies have shown that when IP3R is blocked, low cytoplasmic Ca^{2+} can cause the mitochondria to fail to produce energy normally, resulting in insufficient ATP, which in turn activates the AMPK pathway[68,69]. Others believe that the increased intracellular Ca^{2+} could activate the CaMKKβ and its substrate molecule AMPK[70,71]. Further study is warranted to verify if MI interacts directly or indirectly with IP3R and the complete crosstalk between IP3R and lipid homoeostasis in *E. sinensis*.

Conclusion

In summary, dietary MI could promote growth performance and increase the MI deposition in hepatopancreas and the lipid utilisation of crabs fed high (13 %) lipid through optimising lipid metabolism. Dietary MI could also reduce oxidative damage induced by excessive lipid accumulation in the hepatopancreas of *E. sinensis* fed high lipid diet. Moreover, MI may be an important factor in inhibiting the fatty acid synthesis and promoting fatty acid β-oxidation, which may be partly involved in the up-regulation of the IP3R/CaMKKβ/AMPK pathway in *E. sinensis* fed 13 % lipid (Fig. 6). These results can explain how MI improves the lipid utilisation in crabs fed high lipid diet and provide an in-depth understanding of the application for using MI in crabs fed high lipid diet.

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X. B., X. W. and L. C. conceived the experiment; X. B., Z. L. and C. W. formulated the experimental diets; X. B., L. L. and S. L. conducted the experimental trials and laboratory analysis; Q. S. provided experimental materials and analysis tools. X. B. wrote and revised the manuscript; X. W., J. G. Q. and L. C. conducted polish work of this manuscript. All authors contributed to and approved the manuscript.

No potential conflict of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114521001409

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