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Original Research Article

Effects of dietary methionine on the growth and protein synthesis of juvenile Chinese mitten crabs (*Eriocheir sinensis*) fed fish meal-free diets

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ABSTRACT

This study investigated the effects of dietary methionine (Met) on growth performance and protein synthesis in juvenile Chinese mitten crabs (*Eriocheir sinensis*) fed fish meal (FM)-free diets. Three diets free of FM containing 0.48% (LM), 1.05% (MM), and 1.72% (HM) Met were assessed, and the cysteine content in all the diets was adjusted to 0.46%. The control diet contained 35% FM without Met supplementation. Extra lysine was added to all of the FM-free diets to match the lysine level in the control diet. Juvenile *E. sinensis* (800 crabs weighing 0.74 ± 0.01 g each) were fed these four diets for eight weeks, with five replicates for each treatment. Both the LM and HM groups presented lower weight gain than all the other groups did ($P = 0.002$). The survival of the crabs was lower in the LM and HM groups than in the MM group ($P = 0.005$). Compared with those in the other groups, the growth performance of the crabs in the MM group improved, and lipid deposition and protein accumulation increased. These positive outcomes are associated with high protein expression linked to the mammalian target of the rapamycin (mTOR) pathway and low expression of genes and proteins linked to the PRKR-like endoplasmic reticulum kinase (PERK) pathway. The study of Met supplementation has explored the response of the PERK pathway through reducing glutathione (GSH) levels to promote protein synthesis. The injection of Met and L-buthionine-sulfoximine (BSO), an inhibitor of GSH synthesis, suppressed GSH production and altered the expression of genes and proteins related to protein synthesis pathways. This study suggests that Met supplementation in FM-free diets can increase the growth and protein synthesis of *E. sinensis* by modulating specific cellular pathways, particularly the mTOR and PERK pathways.

1. Introduction

Fish meal is a vital source of aquaculture feeds because of its high protein content and balanced amino acid profiles (Gatlin et al., 2007). However, the increasing demand for fish meal (FM) has led to supply shortages and rising costs. Therefore, alternative protein sources for the aquaculture industry are urgently needed (Bu et al., 2023). In recent decades, low-cost terrestrial plant ingredients have gained extensive attention as potential FM substitutes. However, concerns have been raised about the problems associated with the overuse of plant proteins in aquaculture feed, such as growth inhibition, diminished flesh nutritive value, and decreased health

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status in aquatic animals (Hu et al., 2018; Liu et al., 2019). The primary limitation of plant protein sources in replacing FM in diets is the imbalance of essential amino acids (EAAs) (Barrows et al., 2007; Zhang et al., 2012). Although more soybean meal is commonly used as the main protein source in low FM or FM-free diets for various aquaculture species, methionine (Met), an essential amino acid, is often deficient in soybean meal-based diets, leading to poor growth, anorexia, and impaired immunity in animals (Moura et al., 2019; Walton et al., 1982). Therefore, the effects of low dietary Met levels in low FM or FM-free aquaculture feed should be investigated, especially in diets where soybean meal is the main protein source.

Methionine has been proven to be essential for aquatic animal growth and nutrient utilization (Ahmed, 2014). Extra supplementation with Met in diets without FM has shown potential as a practical solution to mitigate the negative impacts of plant-based proteins (Fang et al., 2021). Methionine supplementation in feed can significantly increase the growth and well-being of various aquatic species, such as Pacific white shrimp (*Litopenaeus vannamei*), European sea bass (*Dicentrarchus labrax*), Chinese sucker (*Myxocyprinus asiaticus*), grass carp fry (*Ctenopharyngodon idella*), and turbot (*Scophthalmus maximus* L.) (Chu et al., 2014; Coutinho et al., 2017; Gao et al., 2019; Ji et al., 2022; Zheng et al., 2023). Moreover, with respect to nutrient utilization, Met is pivotal for increasing protein synthesis, which is also essential for the growth and repair of organisms. This effect has been particularly notable in turbot (*S. maximus* L.) and largemouth bass (*Micropterus salmoides*), where the addition of Met to their diets led to improved protein synthesis (Gao et al., 2019; Zhao et al., 2022).

Methionine functions in protein synthesis through various molecular pathways (Johnna et al., 2019). The specific impact of Met in enhancing the mammalian target of rapamycin 1 (mTORC1) signaling pathway, along with the consequent activation of ribosomal protein S6 (S6), underscores its role in protein translation and transcription (Hyde et al., 2003; Kim and Guan, 2011; Laplante and Sabatini, 2012). On the other hand, a deficiency in Met sharply contrasts with its beneficial effects, resulting in the degradation of proteins and an increase in free amino acid levels within muscle cells. This phenomenon is particularly common in turbot (*S. maximus*) and mouse embryonic fibroblasts (Harding et al., 2003; Jiang et al., 2017). Methionine deficiency can trigger the activation of general control nonderepressible 2 kinase (GCN2) and the phosphorylation of PRKR-like endoplasmic reticulum kinase (PERK) (Harding et al., 1999). This activation and phosphorylation leads to the phosphorylation of eukaryotic translation initiation factor 2 (EIF2α) (Harding et al., 1999, 2003). As the phosphorylation of EIF2α is a critical regulatory step in inhibiting protein translation, this cascade of events may serve as a potential mechanism underlying protein degradation triggered by reduced mRNA translation (Cullinan et al., 2003). The insights gathered underscore the significant role of Met in promoting protein synthesis in aquatic animals and delineate the adverse effects stemming from a lack of this essential amino acid. Although the advantages of Met supplementation and its mechanisms of action have been identified, the exact processes by which Met influences protein synthesis in crustaceans and other aquatic species are still not fully understood. Current research, which has largely focused on mammalian models, provides a fundamental understanding and highlights the need for targeted research in aquatic organisms. This would help in elucidating the specific molecular pathways through which Met operates. This knowledge gap inspires our interest in understanding how Met can enhance aquatic health and growth, thereby contributing to more sustainable aquaculture practices.

Eriocheir sinensis, commonly called the Chinese mitten crab, has attracted significant culinary and nutritional interest in Southeast Asia because of its elevated protein levels, distinctive taste, and considerable nutritional benefits (Liu et al., 2021). The subsequent increase in production has increased the demand for crab feed, highlighting the need for optimized feed formulations (Liu et al., 2021). As a result of the decline in FM supply, the aquaculture industry faces a critical challenge in identifying viable FM-independent dietary solutions. Although soybean meal is an alternative, the possibility of completely replacing FM from the diet of *E. sinensis* has not been extensively studied. The adoption of fermented soybean meal, which is characterized by low levels of antinutritional factors, represents a novel approach as a primary protein source. Empirical evidence suggests that growth and crude protein assimilation in *E. sinensis* are adversely impacted when dietary Met concentrations fall below 1% or exceed 1.6%, with an optimal dietary Met concentration of approximately 1.12% (Ye et al., 2010). This observation indicates a shortfall in Met within diets predominantly based on fermented soybean meal, which is insufficient to meet the growth needs of crabs. Therefore, Met insufficiency could be detrimental to protein synthesis in *E. sinensis*, especially during the juvenile stage (Ji et al., 2022; Nedzarek and Czerniejewski, 2021).

This study aims to examine the effects of dietary Met on growth and protein synthesis in *E. sinensis* fed diets predominantly with fermented soybean meal and devoid of FM. Investigating the impact of Met supplementation in such diets can reveal crucial insights, thereby facilitating the enhancement of protein synthesis and overall growth in crustaceans fed FM-free nutritional regimens.

2. Materials and methods

2.1. Animal ethics statement

All experimental procedures involving animals in this research adhered strictly to the Guidelines for the Care and Use of Laboratory Animals from East China Normal University (20201001).

2.2. Experimental diets

Table 1 presents the four experimental diets and their proximate compositions utilized in this study. The crabs were fed one of four diets: a control diet consisting of 35% FM and three FM-free diets formulated with varying Met concentrations: low (LM), medium (MM), or high (HM). The actual Met concentrations were 4.8 g/kg (LM), 10.5 g/kg (MM), and 17.2 g/kg (HM). As 70% of cysteine can be converted into Met in vivo, we balanced the cysteine content to 0.46% in FM-free diets (Milecam et al., 2021). Glycine, a nonessential amino acid, was added to counterbalance fluctuations in the crude protein content of each diet resulting from dietary Met addition (Ji et al., 2020). Coated Met supplied by King Techina Group (Hangzhou, China) was utilized, with a Met purity of 50%, and the remaining 50% of the coated Met consisted of dextrin. The methods used to measure amino acid contents in the diets were based on China National Standards (GB/T 18246-2019). The diets were freeze-dried overnight and then hydrolyzed for 24 h in 6 mol/L HCl at 110 °C, after which the amino acid composition was subsequently analyzed via a Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan). The measured amino acid contents of the four experimental diets are presented in Table 2. The protein sources in the experimental diets included FM, fermented soybean meal, cottonseed meal, corn gluten meal, and chicken meal (Nanjing Baohui Biological Feed Co., Ltd., Nanjing, China). The main lipid sources were fish, soybean, lecithin, and cholesterol. The diet preparation process was guided by a previous study (Liu et al., 2021). All the raw materials were ground, sieved, and thoroughly mixed via a grinding machine to ensure homogeneity.

Table 1
Formulation and chemical proximate composition of the experimental diets (dry matter basis, %).

Item	Experimental diets[1]			
	FM	LM	MM	HM
Ingredients				
Fish meal[2]	35.00	0.00	0.00	0.00
Fermented soybean meal[2]	0.00	30.00	30.00	30.00
Cottonseed meal[2]	0.00	15.00	15.00	15.00
Corn gluten meal[2]	12.00	12.00	12.00	12.00
Chicken meal[2]	12.00	12.00	12.00	12.00
α-Starch	12.00	12.00	12.00	12.00
Fish oil	1.50	4.50	4.50	4.50
Soybean oil	1.00	0.00	0.00	0.00
Lecithin	1.00	1.00	1.00	1.00
Cholesterol	0.50	0.50	0.50	0.50
Choline chloride	0.50	0.50	0.50	0.50
Vitamin premix[3]	3.00	3.00	3.00	3.00
Mineral premix[4]	2.00	2.00	2.00	2.00
Coated lysine	0.00	1.37	1.37	1.37
Butylated hydroxytoluene	0.05	0.05	0.05	0.05
Cellulose	17.45	2.48	1.68	0.88
Carboxymethyl cellulose	2.00	2.00	2.00	2.00
Glycine	0.00	1.60	0.80	0.00
Coated Met	0.00	0.00	1.60	3.20
Proximate compositions				
Ash	8.13	8.24	8.31	8.29
Moisture	12.55	13.20	12.42	13.10
Crude protein	37.77	38.24	38.13	38.19
Crude lipid	8.78	9.13	9.11	9.20

[1] FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively.
[2] Fish meal (crude protein: 67.19%; crude lipid: 8.95%); fermented soybean meal (crude protein: 51.87%; crude lipid: 4.11%); cottonseed meal (crude protein: 56.11%; crude lipid: 1.66%); corn gluten meal (crude protein: 57.54%; crude lipid: 9.02%); and chicken meal (crude protein: 61.28%; crude lipid: 17.24%).
[3] Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamine hydrochloride, 0.15 g; riboflavin, 0.0625 g; 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; and inositol, 1 g. All ingredients were filled with α-cellulose to 100 g (Han et al., 2019).
[4] 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; $ZnSO_4 \cdot 7H_2O$, 0.476 g; $MnSO_4 \cdot 6H_2O$, 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; and Fe-citrate, 1 g. All glucose was diluted with α-cellulose to 100 g (Han et al., 2019).

Table 2
Amino acid composition of the diets (% dry matter).

Amino acid	Experimental diets[1]			
	FM	LM	MM	HM
Alanine	2.57	1.99	2.04	2.03
Arginine	2.08	2.60	2.60	2.60
Asparagine	3.23	3.42	3.60	3.55
Cystine	0.39	0.46	0.45	0.47
Glutamic acid	5.77	6.86	6.99	7.05
Glycine	2.25	3.09	2.48	1.77
Histidine	1.10	0.92	0.96	0.95
Isoleucine	1.60	1.45	1.48	1.49
Leucine	3.35	3.17	3.23	3.20
Lysine	2.42	2.35	2.35	2.35
Methionine	0.81	0.48	1.05	1.72
Phenylalanine	1.65	1.86	1.93	1.86
Proline	2.10	2.11	2.22	2.16
Serine	1.50	1.65	1.71	1.73
Threonine	1.56	1.40	1.43	1.44
Tyrosine	1.01	1.15	1.23	1.18
Valine	1.80	1.63	1.67	1.67

[1] FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively.

Subsequently, oil and deionized water supplemented with choline chloride were incorporated into the mixture. The blend was then compressed into pellets with a diameter of 2.5 mm via a twin-screw extruder (Guangzhou Huagong Optoelectronic Technology Co., Ltd., Guangzhou, China). The pellets were subjected to drying until the moisture content reached <10%. The samples were subsequently sealed in vacuum bags and stored at −20 °C until use.

2.3. Growth trial and sampling

Healthy juvenile crabs were obtained from a local farm in Shanghai, China. The feeding trial was conducted at the experimental station in Huzhou, Zhejiang. Prior to the formal feeding experiment, the crabs were subjected to a five-day acclimation period and were fed commercial diets to adapt to the experimental conditions. Subsequently, 800 crabs, weighing 0.74 ± 0.01 g each, were randomly allocated to twenty 300-L tanks (100 cm × 50 cm × 60 cm), with five tanks for each treatment group and 40 crabs in each tank. We used four arched tiles and five plastic pipes in each tank to reduce aggression among the crabs. We provided diets equivalent to 4% biomass daily at 14:30 and 20:30 for 8 weeks.

The diets were subjected to a water stability test to assess their capacity to maintain structural integrity when submerged in water for a period of 5 h without dispersing. This test was conducted to ensure that the diets could sustain normal feeding conditions for the crabs without disintegration. Feeding observations were conducted 2 h after feeding, and the daily rations were adjusted to ensure that the feed input slightly exceeded the saturation level. After each feeding session, any remaining feed residue or feces were removed from each tank daily. Additionally, 30% of the filtered pond water was exchanged regularly to maintain water quality at a suitable level. Daily monitoring of water quality parameters was carried out to maintain optimal conditions for the crabs. The water quality parameters were 24 to 26 °C, 7.0 mg/L dissolved oxygen (DO), ammonia <0.05 mg/L and pH 7.5 to 8.4 throughout the growth trial.

After the eight-week feeding experiment, all crabs in each tank were starved for 24 h and subsequently weighed and counted to calculate the final body weight (all crabs were weighed), weight gain, specific growth rate, feed intake, feed conversion ratio (FCR), survival rate, and molting rate (Han et al., 2019). Four crabs were randomly chosen from each tank and preserved at −20 °C for subsequent determination of the proximate composition of the whole body. Furthermore, the hepatopancreas and muscle tissues from eight crabs in each experimental group were promptly isolated and stored at −80 °C for further analysis of enzyme activities and gene expression.

The relevant formulas applied to calculate growth performance and feed utilization were as follows:

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

Specific growth rate (%/d) = 100 × [ln(final body weight) - ln(initial body weight)]/56 days.

Survival (%) = 100 × final crab number/initial crab number.

Molting rate (%) = 100 × molting number/final crab number.

Feed intake (g) = total feed weight/final number.

FCR = total feed weight/(final body weight - initial body weight + dead body weight).

2.4. Injection of Met and L-buthionine-sulfoximine (BSO)

A combined injection experiment with Met and BSO, which are known inhibitors of γ-glutamylcysteine synthetase (γ-GCS), was conducted. This approach was adopted because of ongoing

debates regarding the role of Met in inhibiting the PERK pathway through reduced glutathione (GSH) to stimulate protein synthesis. Juveniles of *E. sinensis* (96 crabs weighing 5.34 ± 0.03 g each) were obtained from a local farm in Shanghai, China, and transported to the laboratory. Before the injection, the crabs were acclimatized to laboratory conditions in 200-L tanks filled with aerated and dechlorinated water and fed commercial diets for 2 weeks. All crabs were then selected and divided into four groups with 24 crabs per group (8 crabs per tank). Phosphate-buffered saline (PBS) was administered to the control (Con) group, whereas the Met group received an injection of 0.2 μg/g Met (purity >98%) (Sigma–Aldrich, Michigan, USA). The BSO group was injected with 0.65 μmol/g BSO (ATCC, Rockville, MD, USA). Finally, the combined group (Met + BSO) received an injection of 0.2 μg/g Met and 0.65 μmol/g BSO. Phosphate-buffered saline was used as a solvent for Met and BSO (Chen et al., 2021; Yu et al., 2017). The crabs in each group received a second injection after a 12-h interval. At 24 h postinjection, 17 crabs were randomly selected from each group ($n = 17$). Samples of the hepatopancreas and muscle were collected for the assessment of GSH content and γ-GCS activity ($n = 6$), as well as for RNA extraction ($n = 8$). Additionally, samples of the hepatopancreas were collected for western blot analysis ($n = 3$). The postinjection sampling time was selected on the basis of previous studies and our preliminary trial (Chen et al., 2021; Yu et al., 2017).

2.5. Proximate biochemical composition analysis

The crude protein content in the diets was determined via the Kjeldahl method (method 988.05; AOAC, 1995), and the crude protein content in the whole body, hepatopancreas, and muscle was determined via the Kjeldahl method (method 981.10) according to the AOAC (1995). The determination of crude lipids in the diet, whole body and hepatopancreas was conducted following the traditional Soxhlet extraction method with diethyl ether (method 920.39; AOAC, 1995). The ash contents in the diets and whole bodies were determined by subjecting the samples to a muffle furnace (F6030CM-33; Thermolyne, Massachusetts, USA) at 550 °C for 6 h (method 924.05; AOAC, 1995). The whole body was subsequently dried in an oven (Thomas Scientific, New Jersey, USA) at 105 °C until a constant weight was reached for analysis of moisture (method 934.01; AOAC, 1995). Each parameter was analyzed in quadruplicate.

2.6. Enzyme activity assay

Hepatopancreas and muscle samples were weighed and subsequently homogenized with saline solution. Six samples per group were used in the experiments. The resulting supernatant was preserved at −80 °C for subsequent analysis. Triglyceride levels were determined via the glycerophosphate oxidase–peroxidase method (Cat. no. A110-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The glutathione content was measured via a colorimetric assay kit (Cat. No. A006-2-1), where GSH reacts with dithiodinitrobenzoic acid to produce a yellow compound measurable at 405 nm. The activity of γ-GCS was assessed via a kit (Cat. no. A091-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In this assay, under the catalysis of γ-GCS, glutamate and cysteine consume adenosine triphosphate, yielding γ-glutamyl cysteine, inorganic phosphorus, and adenosine diphosphate. The amount of inorganic phosphorus produced was quantified at 636 nm.

2.7. Gene expression related to the mTORC1, GCN2 and PERK pathways

According to the manufacturer's instructions, total RNA was extracted from the hepatopancreas and muscle using MagZol reagent (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China). Eight samples per group were included in the experiment. The concentration and quality of the total RNA were assessed via a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The RNA was subsequently used as a template for cDNA synthesis via the PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan). Real-time quantitative PCR (RT–qPCR) was performed on a CFX96 Real-Time PCR system (Bio-Rad, Richmond, USA). The relative expression levels of the target genes were quantified via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with β-actin used as the housekeeping gene. The specific sequences of primers used in this study are listed in Table 3.

2.8. Western blotting

The immunoblot analyses were conducted following our laboratory's established protocol (Lu et al., 2019). Protein homogenates were prepared from hepatopancreas tissues via cell lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China). After centrifugation at 10,400 × *g* for 10 min, the supernatant was mixed with 5 × sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample loading buffer and boiled at 95 °C for 5 min. Protein concentrations were determined via the Pierce Bicinchoninic Acid Protein Assay Kit provided by Thermo Scientific (Massachusetts, USA). Proteins (20 to 50 μg) were subsequently separated via SDS–PAGE and electrophoretically transferred onto nitrocellulose filter membranes. The membranes were then blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 and incubated overnight at 4 °C with primary antibodies against mTOR (66888-1-Ig; Thermo Fisher Scientific, Massachusetts, USA), phosphorylated mTOR (p-

Table 3
Primer pair sequences for quantitative real-time PCR used in this study.

Gene	Position	Primer sequence (5' to 3')	Reference
mtorc1	Forward	AGAAGCTGCATGACTGGGAC	c148249_g1
	Reverse	CGGTCACACGACACACTGTA	
s6k1	Forward	GCACCAGGCTTATTCGACCT	c74214_g1
	Reverse	GGGTTGACTGTGCTGTCTGA	
s6	Forward	TTCCGAGGGTGAACAAGACG	c141087_g1
	Reverse	CTGGCCCATACGCTTCTCAT	
4ebp1	Forward	CAAGGCTGAGCAGGACTTCA	c114480_g1
	Reverse	AGCTGATCCAGGTCACAAGC	
eif4e	Forward	CAAGGCTGAGCAGGACTTCA	c114480_g1
	Reverse	AGCTGATCCAGGTCACAAGC	
gcn2	Forward	CCAAAGGGTTCAAGAAATCA	XM_050870865.1
	Reverse	GAGCGGCGAGGTCAGTA	
perk	Forward	TACCTCTACGCGGATGACAC	XM_033900715.1
	Reverse	TGTGCATGATCTGTGACGTG	
eif2α	Forward	GCCACAATGCCGCTCTT	XP_027214002.1
	Reverse	GCTCCGAACCGTCACCA	
atf4	Forward	GCGCTCTTCACGAAACCC	RLQ72395.1
	Reverse	GGGCTTTGCTGGATTCGAGG	
Beta-actin	Forward	TCGTGCGAGACATCAAGGAAA	KM244725.1
	Reverse	AGGAAGGAAGGCTGGAAGAGTG	

mtorc1 = mammalian target of rapamycin 1; *s6k1* = ribosomal s6 protein kinase 1; *s6* = ribosomal protein s6; *4ebp1* = eukaryotic translation initiation factor 4E-binding protein 1; *eif4e* = eukaryotic translation initiation factor 4E; *gcn2* = general control nonderepressible 2 kinase; *perk* = PRKR-like endoplasmic reticulum kinase; *eif2α* = eukaryotic translation initiation factor 2; *atf4* = activating transcription factor 4.

mTOR) (67778-1-Ig), PERK (ab254249; Abcam plc, Cambridge, UK), phosphorylated PERK (p-PERK) (ab192591), phosphorylated EIF2α (p-EIF2α) (ab32157), GCN2 (ab302609), and β-actin (ab302609). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse proteins. Western blot images were captured via an Odyssey CLx Imager (LICOR, Nebraska, USA), and subsequent quantification of target proteins was conducted via ImageJ 1.44p software (US National Institutes of Health, Bethesda, USA).

2.9. Calculation and statistical analysis

All statistical analyses were conducted using SPSS 20.0 software (Chicago, IL, USA). Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. One-way analysis of variance (ANOVA) was used to assess the differences among the groups. When a significant effect was found, Duncan's multiple range test was applied to compare means at a significance level of $P < 0.05$. The results were expressed as the means and standard error of the mean (SEM).

The statistical model used for the ANOVA is expressed as follows:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

whereY_{ij} represents the observed value for the dependent variable, α_i is the effect of the ith treatment (i.e., different dietary methionine levels), ε_{ij} is the random error term associated with the jth observation under the ith treatment.

In addition to the ANOVA, independent t-tests were used to compare the FM group with the other treatment groups individually. Data from the injection test were analyzed using independent t-tests as well. A significance level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth performance

Compared with those in the FM group, the crabs in the LM and HM groups presented significantly lower final body weights, specific growth rates, and weight gains (Table 4). The FI in the MM group was lower than that in the FM group, and the crabs in the LM group presented a greater FCR than those in the FM group did ($P = 0.013$; Table 4). Conversely, the final body weight, specific growth rate, and weight gain of the crabs fed a FM-free diet with a medium level of dietary Met were greater than those of the crabs fed a FM diet ($P = 0.022$; Table 4). Compared with those fed a FM-free diet with a medium level of dietary Met, those fed FM-free diets with low or high levels of dietary Met tended to have decreased final body weight, weight gain, specific growth rate, and survival ($P = 0.012$, Table 4). Consequently, compared with those fed an FM-free diet with a medium dietary Met level, the feed intake and FCR of the crabs fed FM-free diets with low or high dietary Met levels were greater ($P = 0.028$; Table 4). Conversely, the crabs in the LM and HM groups presented significantly lower molting rates than those in the FM group did ($P = 0.017$; Table 4). The MM group presented a greater molting rate than the LM and HM groups did (Table 4).

3.2. Whole-body, hepatopancreas and muscle proximate composition and hepatopancreas triglyceride content

Among the crabs fed FM-free diets, the MM groups presented the highest values of crude lipid content and crude protein content in the whole body and crude lipids in the hepatopancreas (Table 5). Compared with that in the FM group, the crude protein content of the hepatopancreas was significantly lower in the LM group ($P = 0.034$; Table 5), whereas the crude protein content of the muscle was lower in the HM group ($P < 0.001$; Table 5). However, compared with those in the FM group, the crude protein contents of the hepatopancreas and muscle increased in the MM group ($P = 0.012$; Table 5). In both the hepatopancreas and muscle, the crude protein content of the crabs fed an FM-free diet with a medium level of dietary Met was significantly greater than that of the crabs fed FM-free diets with low or high levels of dietary Met (Table 5).

3.3. Protein synthesis

3.3.1. Gene expression of the mTORC1 pathway

The mRNA expression of genes associated with the mTORC1 pathway in the hepatopancreas and muscle of *E. sinensis* is shown in Fig. 1. Compared with those in the FM group, the expression of *mtorc1* in the hepatopancreas and *s6* in both the hepatopancreas and muscle were upregulated in the MM group ($P = 0.032$; Fig. 1A–C, and H). The gene expression of *mtorc1* and *s6* in the muscle of crabs in the LM group was lower than that in the FM

Table 4
The growth performance of *E. sinensis* fed a fish meal (FM) diet or a FM-free diet supplemented with different levels of dietary methionine (mean ± SEM).

Item	Experimental diets[1]				*P*-value		
	FM	LM	MM	HM	ANOVA[2]	Liner analysis [3]	Quadrative analysis [4]
Initial wet body weight, g	0.74 ± 0.001	0.75 ± 0.001	0.74 ± 0.002	0.74 ± 0.010	0.248	0.115	0.619
Final body weight, g	2.38 ± 0.051*	2.17 ± 0.052^{a,*}	2.76 ± 0.142^{b,*}	2.15 ± 0.061^{a,*}	0.003	0.840	0.001
Weight gain[5], %	220.55 ± 5.623*	190.46 ± 6.961^{a,*}	270.95 ± 17.192^{b,*}	191.28 ± 7.181^{a,*}	0.002	0.948	0.001
Specific growth rate[5], %/d	2.08 ± 0.033*	1.90 ± 0.042^{a,*}	2.35 ± 0.091^{b,*}	1.91 ± 0.051^{a,*}	0.003	0.864	0.001
Feed intake, g	2.55 ± 0.023*	2.51 ± 0.081^{b}	2.26 ± 0.021^{a,*}	2.47 ± 0.072^{b}	0.002	0.934	0.002
FCR	1.56 ± 0.041*	1.77 ± 0.071^{b,*}	1.30 ± 0.192^{a}	1.76 ± 0.081^{b}	0.005	0.826	0.002
Survival[5], %	90.71 ± 2.702	84.29 ± 1.432^{a}	94.00 ± 1.254^{b}	82.86 ± 1.652^{a}	0.005	0.549	0.002
Molting rate[5], %	164.76 ± 2.522*	146.67 ± 2.731^{a,*}	166.67 ± 8.463^{b}	143.81 ± 4.392^{a,*}	0.048	0.736	0.002

FCR = feed conversion ratio.
Asterisks (*) indicate significant differences between crabs fed FM and FM-free diets ($P < 0.05$).
Within a row, means with different letters indicate significant differences in crabs fed the LM, MN and HM diets ($P < 0.05$).
[1] FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively.
[2] The *P*-value was obtained through one-way analysis of variance (ANOVA) to assess differences among crabs fed FM-free diets with varying levels of dietary methionine.
[3] The *P*-value of linear analysis of crabs fed FM-free diets with varying levels of dietary methionine.
[4] The *P*-value of quadratic analysis of crabs fed FM-free diets with varying levels of dietary methionine.
[5] The weight gain, specific growth rate, survival and molting rate values are presented as the means of five measurements.

Table 5
The body, hepatopancreas and muscle compositions of *E. sinensis* fed a fish meal (FM) diet or FM-free diets supplemented with different levels of dietary methionine (mean ± SEM).

Item	Experimental diets[1]				*P*-value		
	FM	LM	MM	HM	ANOVA[2]	Liner analysis [3]	Quadratic analysis [4]
Whole body composition, % of wet weight							
Moisture	64.65 ± 0.061	64.07 ± 0.273	62.91 ± 0.131	63.75 ± 0.342	0.853	0.460	0.049
Ash	12.26 ± 0.112	11.89 ± 0.184	12.02 ± 0.121	11.89 ± 0.152	0.788	1.000	0.508
Total lipid	3.97 ± 0.301	4.07 ± 0.132^{a}	4.89 ± 0.151^{b}	4.53 ± 0.013^{ab}	0.029	0.090	0.019
Total protein	13.37 ± 0.103	13.27 ± 0.172^{a}	13.76 ± 0.153^{b}	13.44 ± 0.022^{ab}	0.043	0.413	0.044
Hepatopancreas composition, % of wet weight							
Total lipid	34.54 ± 1.824	33.78 ± 1.282^{a}	40.63 ± 1.851^{b}	37.11 ± 1.741^{ab}	0.034	0.201	0.042
Total protein	9.75 ± 0.102*	9.26 ± 0.032^{a,*}	10.09 ± 0.054^{c,*}	9.77 ± 0.101^{b}	0.002	0.007	0.002
Triglyceride, mmol/g prot	2.27 ± 0.203	2.33 ± 0.182	2.02 ± 0.081	2.12 ± 0.111	0.529	0.266	0.262
Muscle composition, % of wet weight							
Total protein	16.82 ± 0.363*	17.05 ± 0.262^{b}	18.05 ± 0.261^{c,*}	15.73 ± 0.112^{a,*}	<0.001	0.006	0.001

Asterisks (*) indicate significant differences between crabs fed FM and FM-free diets with different levels of dietary methionine ($P < 0.05$).
Within a row, means with different letters indicate significant differences in crabs fed LM, MN and HM ($P < 0.05$).
[1] FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively.
[2] The *P*-value was obtained through one-way analysis of variance (ANOVA) to assess differences among crabs fed FM-free diets with varying levels of dietary methionine.
[3] The *P*-value of linear analysis of crabs fed FM-free diets with varying levels of dietary methionine.
[4] The *P*-value of quadratic analysis of crabs fed FM-free diets with varying levels of dietary methionine.

group ($P = 0.023$; Fig. 1F and H). Compared with FM treatment, HM treatment significantly decreased the gene expression of *mtorc1* in both the hepatopancreas and muscle, as well as the gene expression of ribosomal s6 protein kinase 1 (*s6k1*) in the hepatopancreas (Fig. 1A–B and F). Compared with those in crabs fed FM-free diets with low or high levels of dietary Met, the expression of the *mtorc1*, *s6k1*, and *s6* genes in both the hepatopancreas and muscle of crabs fed an FM-free diet with medium levels of dietary Met significantly increased (Fig. 1A–C and 1F-H). Compared with the FM diet, the FM-free diet with a high level of dietary Met had higher eukaryotic translation initiation factor 4E-binding protein 1 (*4ebp1*) and eukaryotic translation initiation factor 4E (*eif4e*) mRNA levels in both the hepatopancreas and muscle of *E. sinensis* ($P = 0.022$; Fig. 1D–E and I-J). Similarly, FM-free diets containing low dietary Met presented higher *eif4e* gene expression in muscle than FM diets did ($P = 0.017$; Fig. 1J). In both the hepatopancreas and muscle, the gene expression of *4ebp1* and *eif4e* in the MM group was downregulated compared with that in the LM and HM groups ($P = 0.026$; Fig. 1D–E, 1I–J).

3.3.2. The expression of genes in the GCN2 and PERK pathways

Compared with that in the FM group, the gene expression of *gcn2* in the hepatopancreas was significantly upregulated in the LM group (Fig. 2A). The gene expression levels of *perk*, *eif2α*, and activating transcription factor 4 (*atf4*) in the muscle of the LM group were greater than those in the FM group ($P = 0.036$; Fig. 2F–H). Compared with those fed the FM diet, those fed the FM-free diet with a medium level of dietary Met presented significantly lower *gcn2* gene expression in the hepatopancreas and *perk* and *eif2α* and *atf4* gene expression in both the hepatopancreas and muscle (Fig. 2A–D, F-H). Compared with those in the FM group, the expression of the *perk* and *atf4* genes in both the hepatopancreas and muscle was downregulated in the HM group ($P = 0.018$; Fig. 2B–D, F, and H). Compared with those in the LM group, the mRNA levels of *gcn2*, *perk*, *eif2α*, and *atf4* in both the hepatopancreas and muscle were lower in the MM and HM groups ($P = 0.029$; Fig. 2A–H).

3.3.3. Protein expression related to protein synthesis

The protein expression related to protein synthesis in the hepatopancreas is shown in Fig. 3A. Similar to the findings from the gene expression analysis, the protein expression levels of mTOR and p-mTOR in the hepatopancreas of the MM group were increased compared with those in the FM group ($P = 0.024$; Fig. 3B and C). Compared with those fed a FM diet, those fed a FM-free diet with a high level of dietary Met exhibit decreased mTOR and p-mTOR protein levels in the hepatopancreas ($P = 0.016$; Fig. 3B and C). The protein levels of mTOR and p-mTOR in the hepatopancreas were greater in the MM group than in the LM group, whereas they were lower in the HM group than in the LM group ($P = 0.041$; Fig. 3B and C). Compared with those in the FM group, PERK, p-PERK, and GCN2 protein expression in the hepatopancreas of the crabs in the LM group was increased ($P = 0.013$; Fig. 3D–F). The PERK, p-PERK, and GCN2 protein levels in the MM and HM groups were lower than those in the FM group ($P = 0.026$; Fig. 3D–F). Crabs fed an FM-free diet with a low level of dietary Met presented higher protein levels of PERK, p-PERK, and GCN2 than did those fed an FM-free diet with medium or high levels of dietary Met ($P = 0.024$; Fig. 3D–F).

3.3.4. GSH content and γ-GCS activity

The hepatopancreas of the crabs fed an FM-free diet with a low level of dietary Met presented a markedly lower GSH content than did those fed an FM diet. In contrast, the GSH content in the muscle of the HM group was significantly greater than that in the FM group (Fig. 4A and B). In the MM and HM groups, the content of GSH in the hepatopancreas and muscle was greater than that in the LM group ($P = 0.021$; Fig. 4A and B). The medium and high levels of dietary Met in FM-free diets significantly increased γ-GCS activity in the hepatopancreas and muscle compared with that in FM diets (Fig. 4C and D). Among the LM, MM, and HM groups, the LM group presented the lowest γ-GCS activity in the hepatopancreas and muscle ($P = 0.034$; Fig. 4C and D).

3.4. Combined injection of Met and BSO

3.4.1. The content of GSH and activity of γ-GCS

The GSH content and γ-GCS activity in the hepatopancreas and muscle of crabs injected with Met or BSO are shown in Fig. 5. The GSH content in the hepatopancreas and muscle of the crabs injected with Met was significantly greater than that in the hepatopancreas and muscle of the crabs injected with PBS (Fig. 5A and B). Similarly, the γ-GCS activity in the hepatopancreas and muscle was notably greater in the Met group than in the PBS group ($P = 0.037$; Fig. 5C and D). Compared with those in the Met group, the GSH content and activity of γ-GCS in the hepatopancreas and muscle

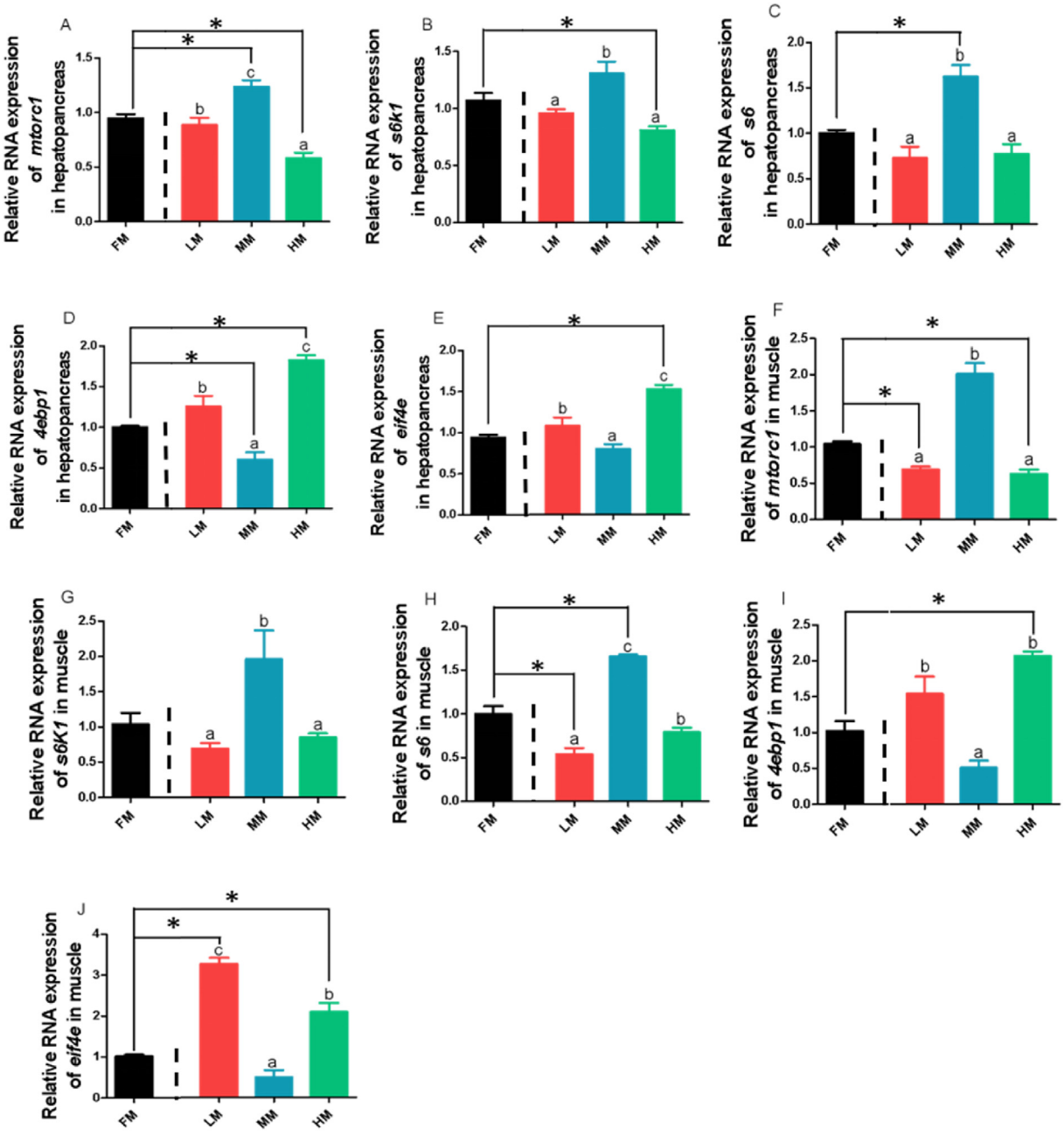

Fig. 1. Gene expression levels of key components involved in the mammalian target of rapamycin 1 (mTORC1) signaling pathway in the hepatopancreas and muscle of *E. sinensis* fed fish meal (FM) diets and FM-free diets containing varying levels of dietary methionine for 8 weeks. *mtorc1*= mammalian target of rapamycin 1; *s6k1* = ribosomal s6 protein kinase 1; *s6* = ribosomal protein s6; *4ebp1* = eukaryotic translation initiation factor 4E-binding protein 1; *eif4e* = eukaryotic translation initiation factor 4E. FM, fish meal diet; LM, MM, or HM, FM-free diet with a low, medium, or high level of dietary methionine, respectively. Different letters on the bars indicate significant differences among crabs fed FM-free diets with different levels of dietary methionine. Asterisks (*) indicate significant differences between crabs fed FM diets and FM-free diets with different levels of dietary methionine.

decreased after simultaneous injection of Met and BSO ($P = 0.005$; Fig. 5A–D).

3.4.2. Gene expression of the GCN2 and PERK pathways

The mRNA levels of *gcn2* in the hepatopancreas and muscle were lower in the Met and Met + BSO groups than in the PBS group ($P = 0.027$; Fig. 6A and E). The gene expression of *perk*, *eif2α*, and *atf4* in the Met group was significantly lower than that in the hepatopancreas and muscle of the PBS group (Fig. 6B–D and F–H). However, crabs simultaneously injected with Met and BSO presented higher levels of *perk*, *eif2α*, and *atf4* gene expression in the hepatopancreas and muscle than those injected with Met alone did ($P = 0.003$; Fig. 6B–D and F–H).

3.4.3. Protein expression of the PERK and GCN2 pathways

The protein levels of the constituents involved in the PERK and GCN2 pathways in the hepatopancreas following combined injection are shown in Fig. 7A. The protein expression levels of PERK, p-PERK and p-EIF2α in the Met group were lower than those in the PBS group ($P = 0.015$; Fig. 7B–D). Compared with those in the Met group, the protein levels of PERK, p-PERK and p-EIF2α in the hepatopancreas increased after BSO injection or simultaneous

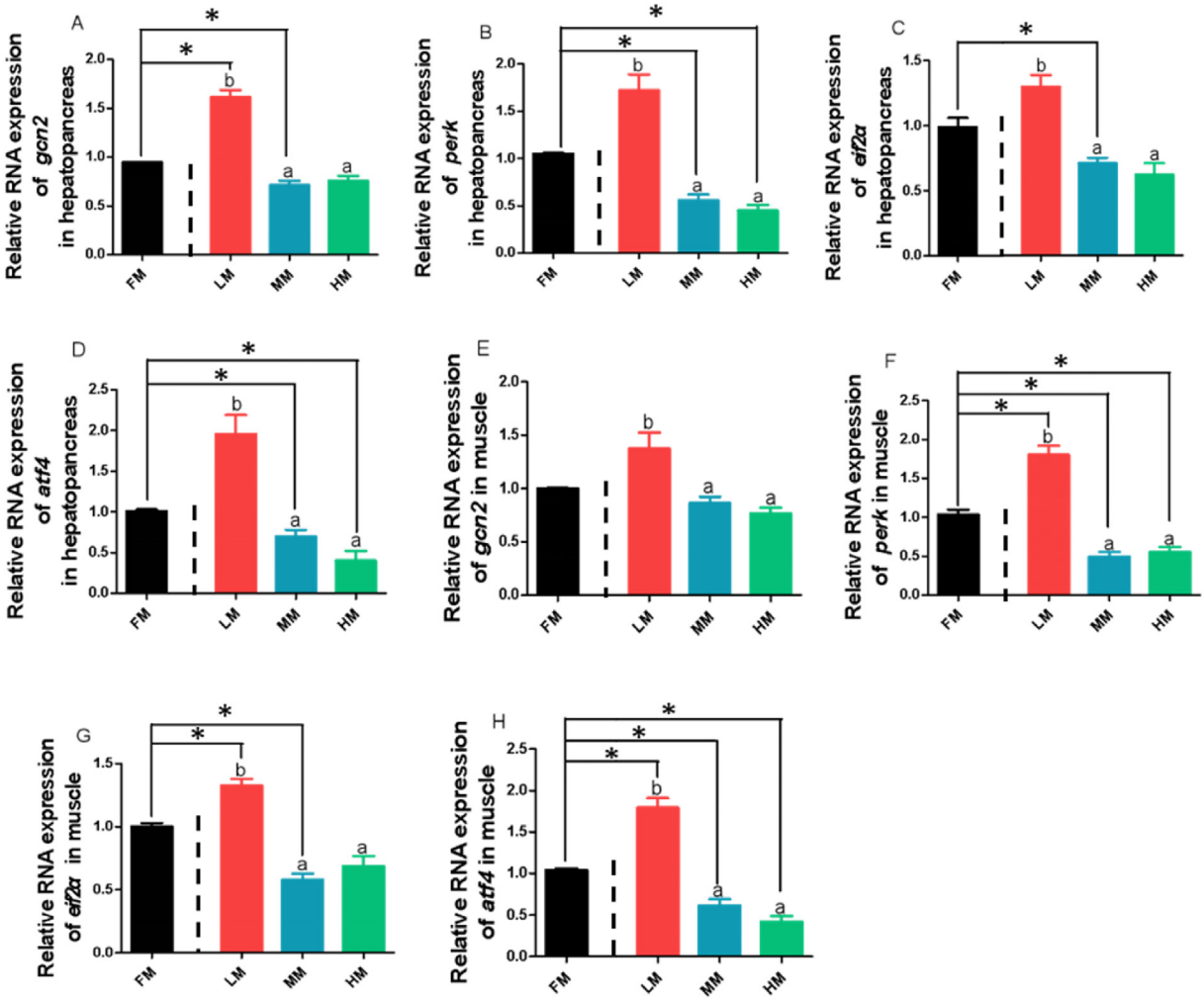

Fig. 2. Gene expression levels of key components involved in the GCN2 and PERK pathways in the hepatopancreas and muscle of *E. sinensis* fed fish meal (FM) and FM-free diets containing varying levels of dietary methionine for 8 weeks. GCN2 = general control nonderepressible 2 kinase; *perk* = PRKR-like endoplasmic reticulum kinase; *gcn2* = general control nonderepressible 2 kinase; *eif2α* = eukaryotic translation initiation factor 2; *atf4* = activating transcription factor 4. FM, fish meal diet; LM, MM, or HM, FM-free diet with a low, medium, or high level of dietary methionine, respectively. Different letters on the bars indicate significant differences among crabs fed FM-free diets with different levels of dietary methionine. Asterisks (*) indicate significant differences between crabs fed FM diets and FM-free diets with different levels of dietary methionine.

injection of Met and BSO ($P = 0.024$; Fig. 7B–D). Compared with that in the PBS group, the protein level of GCN2 in the hepatopancreas significantly decreased in both the Met and Met + BSO groups (Fig. 7E).

4. Discussion

This study investigated the significance of dietary Met in FM-free diets for *E. sinensis*, uncovering its pivotal role in crucial parameters such as survival, weight gain, feed intake, and the feed conversion ratio. Notably, a medium level of dietary Met (1.05%) in the FM-free diets emerged as particularly beneficial, as it improved both survival and weight gain while concurrently reducing feed intake and the feed conversion ratio. These outcomes support the findings from investigations on Pekin ducks, where increasing dietary Met levels were associated with decreased average daily feed intake, indicating the potential of Met to optimize feed efficiency (Wu et al., 2019). These findings underscore the importance of Met, an essential amino acid, in mitigating nutritional imbalances that may otherwise prompt increased feed consumption. Accordingly, this study highlighted the role of Met as a limiting amino acid in FM-free diets for *E. sinensis*, highlighting its indispensable nature for optimal growth and overall health. This assertion is reinforced in research on another crustacean species, *L. vannamei*, where dietary Met supplementation demonstrated growth-promoting effects via the regulation of the somatotropic axis, particularly the growth hormone/insulin-like growth factor-1 pathway (Zheng et al., 2023). Consequently, an FM-free diet containing 1.05% Met emerged as a favorable regimen for fostering the growth of juvenile *E. sinensis*.

The growth of aquatic organisms, such as *E. sinensis*, hinges on the balance between nutrient anabolism and catabolism, facilitating the synthesis and deposition of nutrients within the body (Wu and Morris, 1998). A medium level of dietary Met notably bolstered the crude lipid content in both the hepatopancreas and the entire body, whereas the triglyceride content remained unaffected. This nuanced observation suggests that dietary Met may selectively increase the accumulation of other lipid forms, such as phospholipids, thus revealing a multifaceted role in lipid metabolism (Aissa et al., 2014). Moreover, Met supplementation effectively reversed the decline in protein content, which was evident in FM-free diets, particularly in the hepatopancreas and muscle. This finding aligns with findings demonstrating that dietary Met promotes protein deposition in the entire subadult *S. maximus* L. body, thereby highlighting a promising association between dietary Met

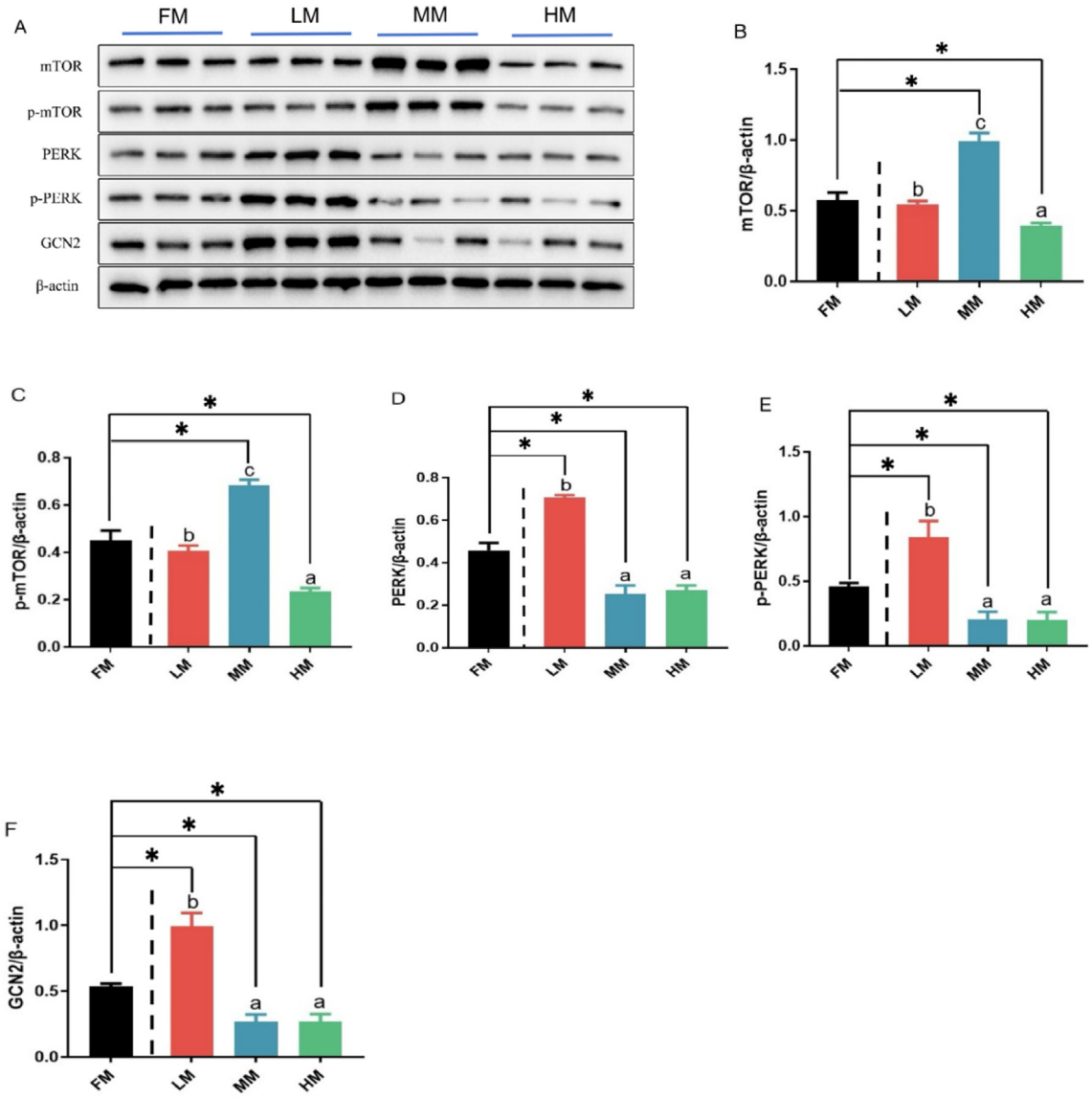

Fig. 3. Protein expression levels of mTOR, p-mTOR, PERK, p-PERK, and GCN2, along with their normalization to β-actin, in the hepatopancreas of *E. sinensis* fed fish meal (FM) and FM-free diets containing varying levels of dietary methionine for 8 weeks. mTOR = mammalian target of rapamycin; p-mTOR = phosphorylated mammalian target of rapamycin; PERK = PRKR-like endoplasmic reticulum kinase; p-PERK = phosphorylated PRKR-like endoplasmic reticulum kinase; GCN2 = general control nonderepressible 2 kinase. FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively. Different letters on the bars indicate significant differences among crabs fed FM-free diets with different levels of dietary methionine. Asterisks (*) indicate significant differences between crabs fed FM diets and FM-free diets with different levels of dietary methionine.

and protein deposition (Sui et al., 2023). Conversely, incorporating excessive dietary Met into FM-free diets reduced the crude protein content within the hepatopancreas and muscle. While essential for fulfilling growth demands, abundant dietary Met engenders cysteine accumulation, leading to oxidative stress and potentially hampering protein synthesis (Toue et al., 2006). Hence, the increase in nutrient accumulation within the body, owing to dietary Met supplementation in FM-free diets, holds promise for bolstering crab growth and vitality (Belghit et al., 2014).

The hepatopancreas and muscle serve as pivotal sites for protein synthesis in *E. sinensis* (Wang and Proud, 2006). The mTORC1 pathway, a sophisticated signaling network activated by nutritional elements, particularly amino acids, controls protein synthesis in *E. sinensis*. This pathway integrates signals via key components such as *4ebp1* and *s6k1*, which are crucial for facilitating protein synthesis (Kim et al., 2013). Our investigation revealed the significant influence of dietary Met supplementation in FM-free diets on the mTORC1 pathway in *E. sinensis*. Notably, there was an increase in the expression of genes (*mtorc1*, *s6k1*, and *s6*) and proteins (mTOR and p-mTOR), which is indicative of heightened activation of the mTORC1 pathway. Simultaneously, the mRNA levels of genes implicated in restraining the mTORC1 pathway (*4ebp1* and *eif4e*) were downregulated, further corroborating pathway activation. These findings are consistent with analogous observations in *C. idella*, implying a conserved mechanism of mTORC1 pathway modulation by dietary Met across diverse species (Ji et al., 2022). Research has shown that Met increases S-adenosylMet (SAM) levels, potentially transmitting regulatory signals to mTOR by engaging the SAM sensor upstream of mTORC1 (Gu et al., 2017). This interaction implies a direct nexus between Met supplementation and mTORC1 pathway activation. Thus, our study reveals that a moderate dietary Met level in FM-free diets promotes protein synthesis in the hepatopancreas and muscle of *E. sinensis* through the mTORC1 pathway.

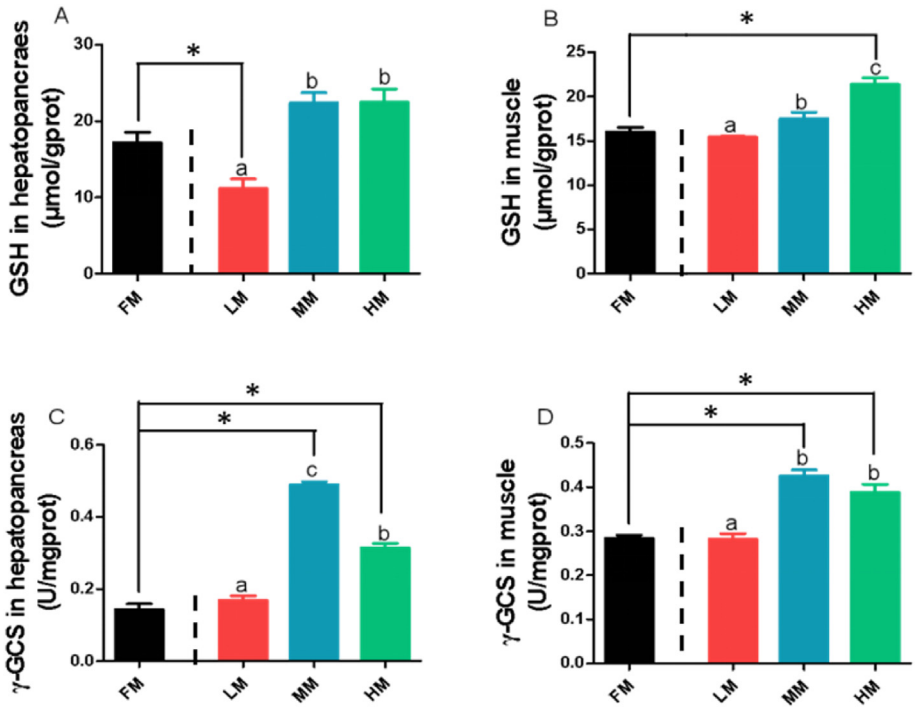

Fig. 4. Glutathione (GSH) content and γ-glutamylcysteine synthetase (γ-GCS) activity in the hepatopancreas and muscle of *E. sinensis* fed fish meal (FM) diets and FM-free diets containing varying levels of dietary methionine for 8 weeks. (A) GSH content in the hepatopancreas of *E. sinensis*. (B) GSH content in the muscle of *E. sinensis*. (C) γ-GCS activity in the hepatopancreas of *E. sinensis*. (D) γ-GCS activity in the muscle of *E. sinensis*. FM, fish meal diet; LM, MM, or HM, fish meal-free diet with a low, medium, or high level of dietary methionine, respectively. Different letters on the bars indicate significant differences among crabs fed FM-free diets with different levels of dietary methionine. Asterisks (*) indicate significant differences between crabs fed FM diets and FM-free diets with different levels of dietary methionine.

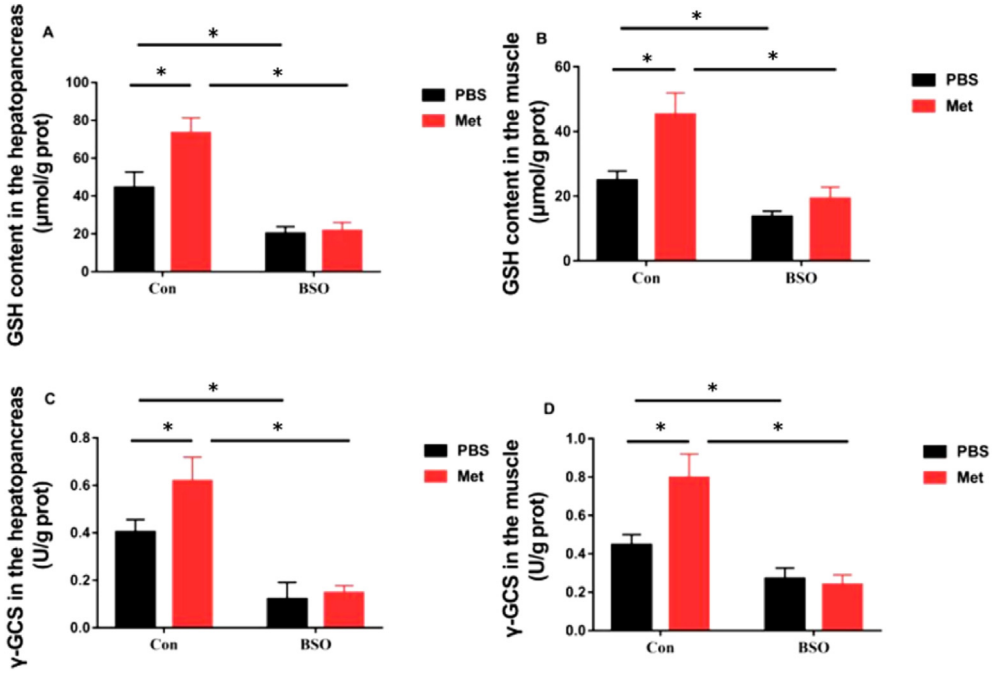

Fig. 5. Effects of Met or BSO injection on the GSH content and γ-GCS activity in the hepatopancreas and muscle of *E. sinensis* after 24 h. **(A)** GSH content in the hepatopancreas of *E. sinensis*. **(B)** GSH content in the muscle of *E. sinensis*. **(C)** γ-GCS activity in the hepatopancreas of *E. sinensis*. **(D)** γ-GCS activity in the muscle of *E. sinensis*. Con = the control group; BSO = L-buthionine-sulfoximine; GSH = glutathione; PBS = phosphate-buffered saline; Met = methionine. Asterisks (*) indicate significant differences between different treatments.

Protein synthesis in mammals is finely regulated by pathways other than mTORC1, notably the GCN2 and PERK signaling pathways (Donnelly et al., 2013). These pathways play crucial roles in cellular responses to essential amino acid deficiency, with Met deficiency specifically triggering the activation of GCN2 and PERK (Cullinan and Diehl, 2004). This activation leads to downstream

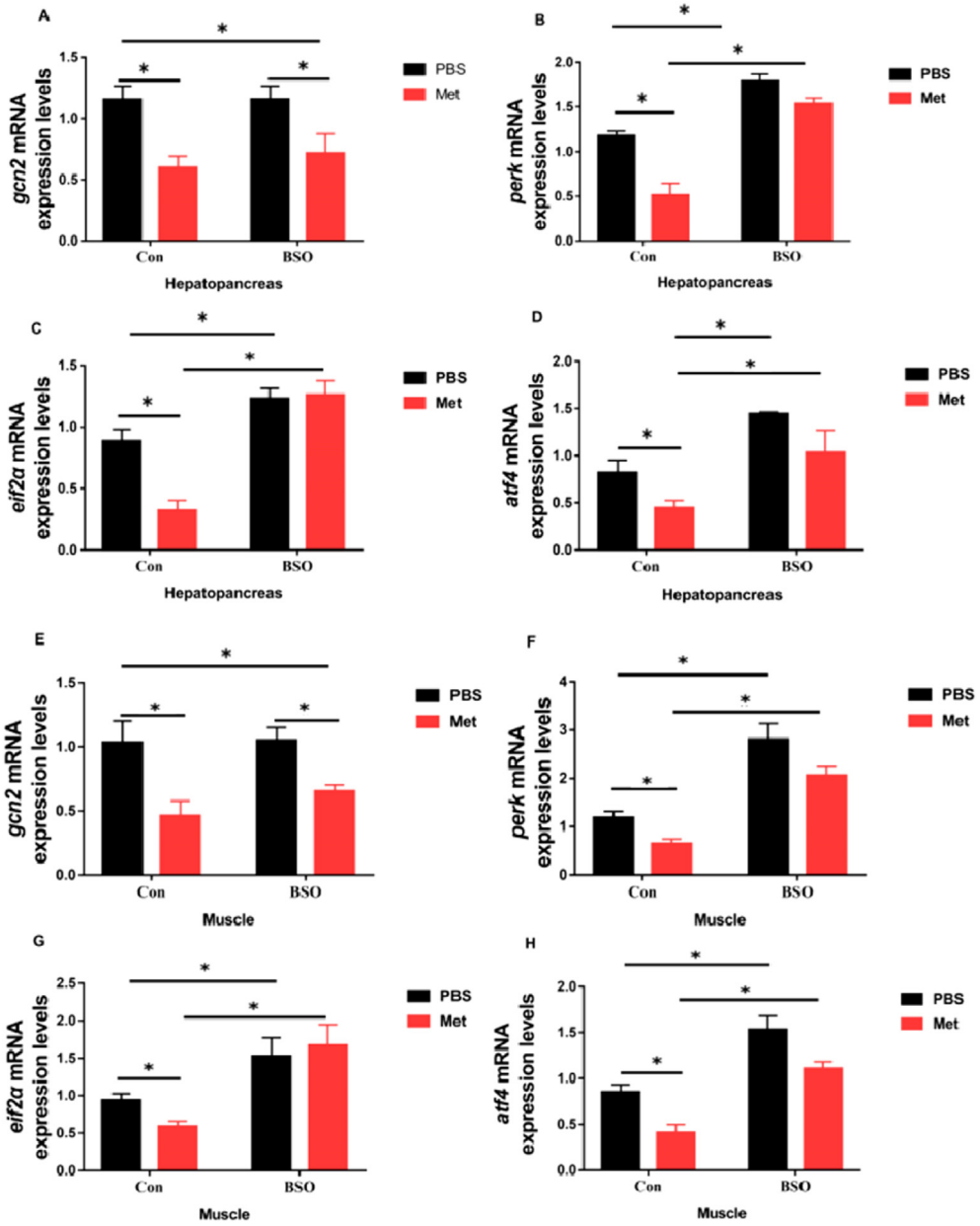

Fig. 6. Gene expression levels of key components involved in the GCN2 and PERK pathways in the hepatopancreas and muscle of *E. sinensis* 24 h after the injection of Met or BSO. **(A)** Expression levels of *gcn2* in the hepatopancreas of *E. sinensis*. **(B)** The expression levels of *perk* in the hepatopancreas of *E. sinensis*. **(C)** Expression levels of *eif2α* in the hepatopancreas of *E. sinensis*. **(D)** Expression levels of *atf4* in the hepatopancreas of *E. sinensis*. **(E)** Expression levels of *gcn2* in the muscle of *E. sinensis*. **(F)** The expression levels of *perk* in the muscle of *E. sinensis*. **(G)** Expression levels of *eif2α* in the muscle of *E. sinensis*. (H) Expression levels of *atf4* in the muscle of *E. sinensis*. Con = the control group; BSO = L-buthionine-sulfoximine; PBS = phosphate-buffered saline; Met = methionine; GSH = glutathione; γ-GCS = γ-glutamylcysteine synthetase; *gcn2* = general control nonderepressible 2 kinase; *perk* = PRKR-like endoplasmic reticulum kinase; *eif2α* = eukaryotic translation initiation factor 2; *atf4* = activating transcription factor 4. Asterisks (*) indicate significant differences between different treatments.

phosphorylation of eIF2α and subsequent activation of ATF4, mechanisms critical for dampening protein synthesis (Donnelly et al., 2013). In our study, moderate dietary Met levels in FM-free diets downregulated the mRNA expression of *gcn2* and *perk*, along with the protein expression of GCN2, PERK, and p-PERK, resulting in the inhibition of their downstream molecules *eif2α*/*atf4*. Additionally, dietary Met inhibits stress-induced pathways and facilitates the synthesis of GSH. The activity of γ-GCS, the rate-limiting enzyme in the synthesis of GSH from Met, increased in crabs fed FM-free diets containing medium and high levels of dietary Met (Donnelly et al., 2013). This increase in enzyme activity elevated the GSH levels in the hepatopancreas and muscle. While an imbalance in essential amino acids typically activates PERK, it can also be induced by a reduction in GSH levels concentrated in the endoplasmic reticulum (Cullinan and Diehl, 2004). Overall, the findings of our study suggested that dietary Met may inhibit the GCN2 pathway (GCN2/*eif2α*/*atf4*) while simultaneously being metabolized to GSH to inhibit the PERK pathway (PERK/*eif2α*/*atf4*), with both pathways collectively regulating protein synthesis.

This study hypothesizes that dietary Met influences protein synthesis in *E. sinensis* by metabolizing GSH, a critical antioxidant. Glutathione synthesis, facilitated by γ-GCS, can be inhibited by BSO, providing a method to explore the role of Met in protein synthesis regulation (Gallagher and Di, 1992). A combined injection

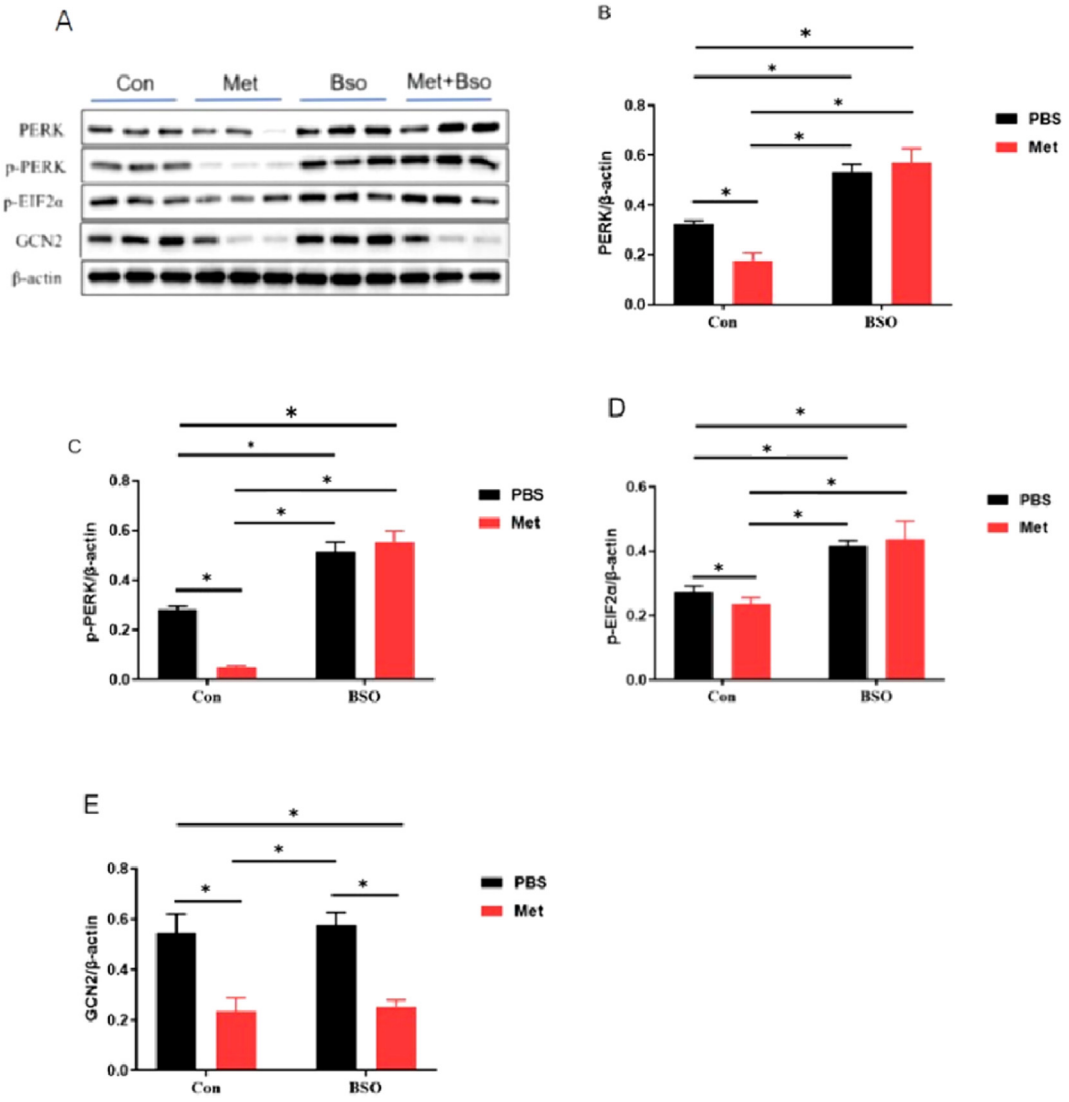

Fig. 7. Protein expression levels of PERK, p-PERK, p-EIF2α, and GCN2, along with their normalization to β-actin in the hepatopancreas of *E. sinensis* 24 h after the injection of Met or BSO. **(A)** The protein levels of PERK, p-PERK, p-EIF2α, GCN2 and β-actin in the hepatopancreas of *E. sinensis*. **(B)** The relative protein expression levels of PERK in the hepatopancreas of *E. sinensis*. **(C)** The relative protein expression levels of p-PERK in the hepatopancreas of *E. sinensis*. **(D)** The relative protein expression levels of p-EIF2α in the hepatopancreas of *E. sinensis*. **(E)** The relative protein expression levels of GCN2 in the hepatopancreas of *E. sinensis*. Con = the control group; BSO = L-buthionine-sulfoximine; PBS = phosphate-buffered saline; Met = methionine; PERK = PRKR-like endoplasmic reticulum kinase; p-PERK = phosphorylated PRKR-like endoplasmic reticulum kinase; p-EIF2α = eukaryotic translation initiation factor 2; GCN2 = general control nonderepressible 2 kinase. Asterisks (*) indicate significant differences between different treatments.

experiment using Met and BSO was conducted. This approach aimed to assess how the inhibition of GSH synthesis impacts protein synthesis pathways in *E. sinensis*. The results of injection with Met and BSO confirmed that the GSH content and γ-GCS activity in the hepatopancreas and muscle simultaneously decreased in *E. sinensis*, indicating that the biosynthesis of GSH from Met was effectively disrupted by BSO (Gallagher and Di, 1992).

The gene expression levels of *perk*, *eif2α*, and *atf4* in the hepatopancreas and muscle and the protein levels of PERK, p-PERK, and p-EIF2α in the hepatopancreas were significantly increased when crabs were injected with Met and BSO, demonstrating that the activation of the PERK pathway is not solely related to Met deficiency but is also mediated by a reduction in GSH.

Interestingly, while Met and BSO injections inhibited GCN2 expression, they increased the expression of *eif2α* and *atf4* and the protein level of p-EIF2α. Similarly, the absence of GCN2 did not affect the ability of dietary Met restriction to activate EIF2α protein expression in mice, suggesting that GCN2 may not be a direct upstream target of EIF2α (Saxton et al., 2016). Finally, the present study demonstrated that inhibiting EIF2α/*atf4* in the hepatopancreas and muscle of *E. sinensis* caused by dietary Met can be independent of GCN2. However, further studies are needed to verify the regulatory relationship between GCN2 and EIF2α/*atf4*. Therefore, this study suggests that dietary Met can improve protein synthesis in *E. sinensis* fed FM-free diets by activating the mTORC1 pathway and potentially inhibiting the GSH-mediated PERK pathway.

5. Conclusions

The inclusion of 1.05% Met in FM-free diets improved growth performance, including increased weight gain and survival. Moreover, it facilitates the accumulation of lipids (excluding triglycerides) in the whole body and hepatopancreas by enhancing lipid metabolism. Dietary Met induces protein deposition in various tissues, including the whole body, hepatopancreas, and muscle, demonstrating its importance in protein synthesis. Furthermore,

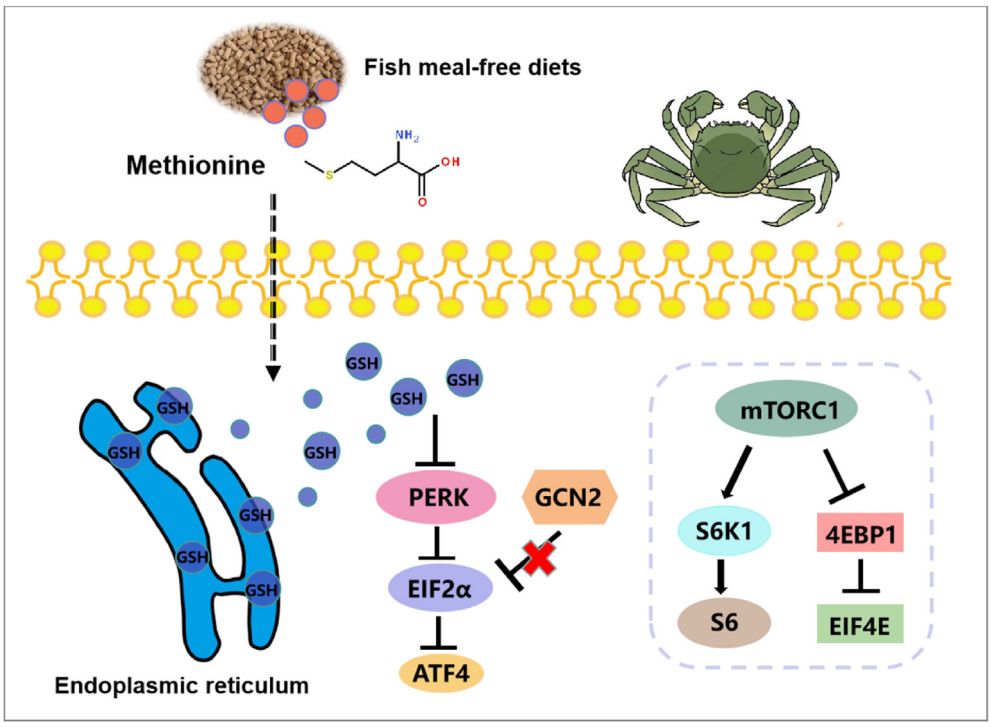

Fig. 8. Schematic representation illustrating the proposed model of dietary methionine supplementation in fish meal-free diets, inducing protein gain in the hepatopancreas and muscle through the mTORC1 and PERK pathways. Key components of the pathways are indicated. mTORC1 = mammalian target of rapamycin 1; S6K1 = ribosomal s6 protein kinase 1; S6 = ribosomal protein s6; 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1; EIF4E = eukaryotic translation initiation factor 4E; GCN2 = general control nonderepressible 2 kinase; PERK = PRKR-like endoplasmic reticulum kinase; EIF2α = eukaryotic translation initiation factor 2; ATF4 = activating transcription factor 4.

this study provides insights into the molecular mechanisms underlying the effects of dietary Met on protein synthesis regulation (Fig. 8). This finding suggests that dietary Met may contribute to the upregulation of the mTORC1 pathway in the hepatopancreas and muscle, thereby promoting protein synthesis. Methionine supplementation may preferentially promote the production of GSH. This shift in signaling pathways influences the downstream PERK/EIF2α/*Atf4* pathway, ultimately leading to increased protein synthesis.

Credit author statement

Jiadai Liu did the experiment, analysed data and drafted manuscript. **Erchao Li** and **Liqiao Chen** supervised the research and polished the manuscript. **Xinyu Li**, **Qincheng Huang**, **Han Wang**, and **Yixin Miao** helped with the farming, sampling process and indicator measurements. **Xiaodan Wang**, **Qingchao Shi** and **Jianguang Qin** helped manuscript writing and data interpretation.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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References

Ahmed I. Dietary amino acid l-methionine requirement of fingerling Indian catfish, *Heteropneustes fossilis* (Bloch-1974) estimated by growth and haematobiochemical parameters. Aquacult Res 2014;45:243–58.
Aissa AF, Tryndyak V, Conti A, Melnyk S, Gomes TDUH, Bianchi MLP, James SJ, Beland FA, Antunes LMG, Pogribny IP. Effect of methionine-deficient and methionine-supplemented diets on the hepatic one-carbon and lipid metabolism in mice. Mol Nutr Food Res 2014;58:1502–12.
AOAC (Association of Official Analytical Chemists). Official methods of analysis of official chemists international. 16th ed. Arlington, VA: Association of Official Analytical Chemists; 1995. p. 3–5.
Barrows FT, Stone DAJ, Hardy RW. The effects of extrusion conditions on the nutritional value of soybean meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2007;265:244–52.
Belghit I, Skiba CS, Geurden I, Dias K, Surget A, Kaushik S, Panserat S, Seiliez I. Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (*Oncorhynchus mykiss*). Br J Nutr 2014;112:493–503.
Bu XY, Li YR, Lai WC, Yao CW, Liu YT, Wang Z, Zhao ZQ, Han SZ, Du JL, Yao X, Mai KS, Ai QH. Innovation and development of the aquaculture nutrition research and feed industry in China. Rev Aquacult 2023:1–16.
Chen MJ, Zhou JY, Chen YJ, Wang XQ, Yan HC, Gao CQ. The in ovo injection of methionine improves intestinal cell proliferation and differentiation in chick embryos by activating the JAK2/STAT3 signaling pathway. Anim Nutr 2021;7:1031–8.
China National Standard. Determination of amino acids in feeds (GB/T 18246-2019). Beijing: Standards Press of China; 2019.
Chu ZJ, Gong Y, Lin YC, Yuan YC, Cai WJ, Gong SY, Luo Z. Optimal dietary methionine requirement of juvenile Chinese sucker, *Myxocyprinus asiaticus*. Aquacult. Nutrition 2014;20:253–64.
Coutinho F, Simoes R, Monge OR, Furuya WM, Pousao FP, Kaushik S, Oliva TA, Peres H. Effects of dietary methionine and taurine supplementation to low-fish meal diets on growth performance and oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 2017;479:447–54.
Cullinan SB, Diehl JA. PERK-Dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. J Biol Chem 2004;279:20108–17.
Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 2003;23:7198–209.
Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2α kinases: their structures and functions. Cell Mol Life Sci 2013;70:3493–511.

Fang CC, Feng L, Jiang WD, Wu P, Liu Y, Kuang SY, Tang L, Liu XA, Zhou XQ. Effects of dietary methionine on growth performance, muscle nutritive deposition, muscle fiber growth and type I collagen gain of on-growing grass carp (*Ctenopharyngodon idella*). Br J Nutr 2021;126:321–36.
Gallagher EP, Di RT. A comparison of glutathione-dependent enzymes in liver, gills and posterior kidney of channel catfish (*ictalurus punctatus*). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1992;102:543–7.
Gao Z, Wang X, Tan C, Zhou H, Mai K, He G. Effect of dietary methionine levels on growth performance, amino acid metabolism and intestinal homeostasis in turbot (*Scophthalmus maximus* L.). Aquaculture 2019;498:335–42.
Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl A, Nelson R, Overturf K, Rust M. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult Res 2007;38:551–79.
Gu X, Orozco JM, Saxton RA, Condon KJ, Liu GY, Krawczyk PA, Scaria SM. SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. Science 2017;358:3–6.
Han F, Wang X, Guo J, Qi C, Xu C, Luo Y, Li E, Qin JG, Chen L. Effects of glycinin and β-conglycinin on growth performance and intestinal health in juvenile Chinese mitten crabs (*Eriocheir Sinensis*). Fish Shellfish Immunol 2019;84:269–79.
Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 1999;397:271–4.
Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003;11:619–33.
Hu HC, Han D, Zhu X, Yang Y, Jin J, Liu H, Xie S. Effect of different protein source diets on growth, sensory parameters and flesh texture of on-growing grass carp (*Ctenopharyngodon idellus*). ISR J Aquacult-Bamid 2018;70.
Hyde R, Taylor PM, Hundal HS. Amino acid transporters: roles in amino acid sensing and signaling in animal cells. Biochem J 2003;373:1–18.
Ji K, Liang H, Ge X, Ren M, Pan LK, Huang D. Optimal methionine supplementation improved the growth, hepatic protein gain and lipolysis of grass carp fry (*Ctenopharyngodon idella*). Aquaculture 2022:1–2.
Ji K, Huang L, Ren M, Ge XP, Mi HF, Pan LK, Yu Heng. The immunoreaction and antioxidant capacity of juvenile blunt snout bream (*Megalobrama amblycephala*) involves the PI3K/Akt/Nrf2 and NF-κB signaling pathways in response to dietary methionine levels. Fish Shellfish Immunol 2020;105:126–34.
Jiang H, Bian F, Zhou H, Wang X, Wang K, Mai K, He G. Nutrient sensing and metabolic changes after methionine deprivation in primary muscle cells of turbot (*Scophthalmus maximus* L.). J Nutr Biochem 2017;50:74–82.
Johnna OZ, Sarah M, Stella CK, Murat D, Ines N, Rose W, Ariane H, Klaus E. Effects of methionine on muscle protein synthesis and degradation pathways in broilers. J Anim Physiol Anim Nutr 2019;103:191–203.
Kim J, Guan KL. Amino acid signaling in TOR activation. Annu Rev Biochem 2011;80(1):1001–32.
Kim SG, Buel GR, Blenis J. Nutrient regulation of the mTOR complex 1 signaling pathway. Mol Cell 2013;35:463–73.
Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell 2012;149:274–93.
Liu S, Wang X, Bu X, Lin Z, Li E, Shi Q, Zhang M, Qin JG, Chen L. Impact of Dietary Vitamin D(3) Supplementation on growth, molting, antioxidant capability, and immunity of juvenile Chinese mitten crabs (*Eriocheir sinensis*) by metabolites and vitamin D receptor. J Agric Food Chem 2021;69:12794–806.
Liu X, Feng L, Jiang WD, Wu P, Jiang J, Tang L, Kuang SY, Zhou XQ, Liu Y. Dimethyl-β-propiothetine (DMPT) supplementation under the all-plant protein diet enhances growth performance, digestive capacity and intestinal structural integrity for on-growing grass carp (*Ctenopharyngodon idella*). Aquaculture 2019;513:734421.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
Lu DL, Ma Q, Wang J. Fasting enhances cold resistance in fish through stimulating lipid catabolism and autophagy. J Physiol-London 2019;597:1585–603.
Milecam J, Khan DR, Dedeurwaerder A, Saremi B. Optimal methionine plus cystine requirements in diets supplemented with L-methionine in starter, grower, and finisher broilers. Poultry Sci 2021;100:910–7.
Moura LB, Diogenes AF, Campelo DAV, Almeida FLA, Pousao FPM, Furuya WM, Peres H, Oliva TA. Nutrient digestibility, digestive enzymes activity, bile drainage alterations and plasma metabolites of meagre (*Argyrosomus regius*) feed high plant protein diets supplemented with taurine and methionine. Aquaculture 2019:734231.
Nedzarek A, Czerniejewski P. The edible tissues of the major European population of the invasive Chinese mitten crab (*Eriocheir sinensis*) in the Elbe River, Germany, as a valuable and safe complement in essential elements to the human diet. J Food Compos Anal 2021;96:103713.
Saxton RA, Chantranupong L, Knockenhauer KE, Schwartz TU, Sabatini DM. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. Nature 2016;536:229–33.
Sui ZM, Wang X, Sun YK, Zhou HH, Liu CD, Mai KS, He G. Optimal dietary methionine requirement of subadult turbot (*Scophthalmus maximus* L.): growth performance, feed utilization and hepatic lipid metabolism. Aquaculture 2023;3:566–7.
Toue S, Kodama R, Amao M, Kawamata Y, Kimura T, Sakai R. Screening of toxicity biomarkers for methionine excess in rats. J Nutr 2006;136:1716–21.
Walton MJ, Cowey CB, Adron JW. Methionine metabolism in rainbow trout fed diets of differing methionine and cystine content. J Nutr 1982;112:1525–35.
Wang X, Proud CG. The mTOR pathway in the control of protein gain. Physiology (Bethesda) 2006;21:362–9.
Wu G, Morris SM. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336:1–17.
Wu YB, Tang J, Xie M, Zhao R, Huang W, Zhang Q, Hou SS. Effects of dietary energy and methionine on growth performance and carcass traits of growing Pekin ducks from 15 to 42 days of age. Poultry Sci 2019;3:5874–5.
Ye JY, Wang YH, Guo KL, Chen JM, Pan Q, Shen BQ. Lysine, methionine and argine requirements of juvenile Chinese mitten crab (*Eriocheir sinensis*). J Fish China 2010;34(10):1544–7.
Yu M, Liu Y, Duan Y, Chen Y, Han J, Sun L, Yang X. Inhibition of glutathione production by L-S,R-buthionine sulfoximine activates hepatic ascorbate gain - a unique antioxidative stress mechanism in mice. Biochem Biophys Res Commun 2017;484:56–63.
Zhang YX, Overland M, Shearer KD, Sorensen M. Optimizing plant protein combinations in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*) by a mixture model. Aquaculture 2012;360–361:25–36.
Zhao Y, Yang C, Zhu XX, Feng L, Liu Y, Jiang WD, Wu P, Huang XL, Chen DF, Yang SY, Luo W, Zhang JX, Li SW, Diao H, Wei XL. Dietary methionine hydroxy analog supplementation benefits on growth, intestinal antioxidant status and microbiota in juvenile largemouth bass *Micropterus salmoides*. Aquaculture 2022;556:738279.
Zheng L, Liu YC, Zhang YM, Xu BY, Sagada G, Wang ZX. Comparative study on the effects of crystalline L-methionine and methionine hydroxy analog calcium supplementations in the diet of juvenile Pacific white shrimp (*Litopenaeus vannamei*). Front Physiol 2023:1–10.