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Original Research Article

Replacement of fish meal with cottonseed protein concentrate in Chinese mitten crab (*Eriocheir sinensis*): Nutrient digestibility, growth performance, free amino acid profile, and expression of genes related to nutrient metabolism

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ABSTRACT

This study aimed to investigate the application of cottonseed protein concentrate (CPC) in Chinese mitten crabs (*Eriocheir sinensis*). First, the apparent digestibility coefficient (ADC) of CPC, fish meal and soybean meal were compared in crabs (21.72 ± 0.33 g). The protein ADC of CPC was 90.42%, which was significantly higher than that of soybean meal (83.16%) ($P < 0.05$). The ADC of Phe, Cys and Glu of CPC were significantly higher than those of fish meal, while the ADC of Ile, Leu, Lys, Met, Thr and Ala of CPC were significantly lower ($P < 0.05$). Second, we investigated the effects of fish meal substitution by CPC on growth performance, free amino acid profile, and expression of genes related to nutrient metabolism in crabs. Six diets were formulated by replacing 0%, 15%, 30%, 45%, 60% and 75% fish meal with CPC, namely FM, CPC15, CPC30, CPC45, CPC60, and CPC75. A total of 630 crabs (1.68 ± 0.00 g) were randomly divided into 18 tanks (3 tanks per group) and fed 3 times daily for 9 weeks. Results showed that CPC75 group significantly reduced growth performance, feed conversion efficiency, and free Ile, Leu, Lys, Met, and Thr contents in muscle ($P < 0.05$). The contents of free amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val, Ala, Cys, Glu, Gly, Ser and Tyr) in hepatopancreas decreased linearly with the increase of dietary CPC level ($P < 0.05$). The substitution of more than 45% fish meal with CPC significantly decreased the concentration of delicious amino acids (Ala, Glu and Gly) in hepatopancreas ($P < 0.05$), which might adversely affect crab flavor. The expression of genes related to antioxidant capacity, protein transport, TOR pathway and lipid metabolism was significantly downregulated by increasing dietary CPC level ($P < 0.05$). In conclusion, based on the quadratic regression analysis of FCR and PER, the optimal replacement levels of fish meal with CPC in crab diet containing 35% fish meal were 32.36% and 35.38%, respectively. It is recommended that Ile, Leu and Thr be supplemented in addition to Met and Lys in the application of CPC.

1. Introduction

The Chinese mitten crab (*Eriocheir sinensis*) is an excellent protein source for human consumption with a unique flavor and high nutritional value. In 2019, the total production of Chinese mitten crabs reached roughly 800,000 tonnes (FAO, 2021), which is an economic pillar of freshwater aquaculture in China and hence the world. The application of formulated feeds in aquaculture has advanced in leaps and bounds to satisfy the nutritional requirements of aquatic animals and achieve scientific farming. It is

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well known that the quality of protein source significantly impacts the growth and health of farm animals. Among the many protein sources, fish meal has the characteristics of high digestibility, balanced amino acid composition, rich in vitamins, minerals and unsaturated fatty acids, and is considered to be the main source of protein in aquatic animal diets. However, in recent years, there has been a shortage of fish meal in the market due to the rapid growth of aquaculture and the stagnant annual worldwide fish meal production (Olsen and Hasan, 2012). It is therefore particularly important to find sustainable protein sources to replace fish meal.

Cottonseed is a plentiful plant protein that is sustainable in most regions of the world, and has a wide range of applications in food, agriculture and medicine industries (Kumar et al., 2021). A newly developed cottonseed product, cottonseed protein concentrate (CPC), has a high crude protein concentration and an excellent balance of amino acids (Xie et al., 2022). Compared with cottonseed meal, a large amount of free gossypol (an anti-nutritional factor unique to cottonseed) is removed from CPC by one-step low-temperature leaching and two-solvent stepwise extraction processes (Xie et al., 2022). Thus, CPC has great potential for application in feed.

Digestibility and utilization are generally important basic indicators of ingredient quality (Glencross, 2020). Evaluating the digestibility of nutrients in ingredients is one of the most effective ways to clarify the nutritional value of an ingredient for animals and its rational use (Glencross, 2020). However, the apparent digestibility coefficient (ADC) of CPC in crustaceans has not yet been determined. The utilization is obtained mainly by assessing feed intake (FI) and weight gain (WG) of the target species after feeding experimental diets to that species (Glencross, 2020). To date, CPC has been widely studied in fish, such as pearl gentian grouper (*Epinephelus drummondhayi*) (Chen et al., 2020; He et al., 2021; Ye et al., 2020; Yin et al., 2018), largemouth bass (*Micropterus salmoides*) (Huang et al., 2017; Xie et al., 2022; Xu et al., 2022), rainbow trout (*Oncorhynchus mykiss*) (Zhao et al., 2021) and golden pompano (*Trachinotus ovatus*) (Qin et al., 2021; Shen et al., 2020). In crustaceans, it has been shown that in a basal diet containing 25% fish meal, CPC could replace up to 45% of fish meal (CPC inclusion levels 0% to 13.16%) without having a negative impact on growth and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) (Wang et al., 2022). CPC could replace 19.07% and 22.73% of fish meal (CPC inclusion levels 8.90% to 10.61%) for swimming crab (*Portunus trituberculatus*) (Xie et al., 2022). Thus, CPC may not be used in high levels in crustacean diet formulations. One of the key reasons for limiting the dietary CPC level may be the contents and availability of amino acids (such as Met and Lys) in the feed after replacement. Therefore, it is particularly important to study the amino acid contents in the replacement diet, as well as the free amino acid levels in tissues and the regulated signaling pathways after animal feeding. However, research in this area is lacking, especially for crustaceans.

Besides serving as substrates for protein synthesis, amino acids are known as signaling molecules involved in cell growth and metabolic regulation (Skiba-Cassy et al., 2016). The target of rapamycin (TOR) is a highly conserved serine/threonine kinase that can be activated by amino acids to regulate cell growth and metabolism (Kim and Guan, 2011). TOR signaling pathway plays a crucial role in regulating protein synthesis in fish and mammals through eukaryotic translation initiation factor 4E (eIF4E), eIF4E binding protein (4EBP) and ribosomal protein S6 kinase (S6K) (Liu and Sabatini, 2020). Therefore, it is necessary to know whether replacement levels are associated with changes in amino acid composition and TOR signaling pathway. Clarifying the mechanisms of protein and amino acid metabolism in animals helps to achieve efficient feed utilization, and is vital for maintaining optimal growth of animals.

Meanwhile, it is shown that TOR signaling pathway can influence the expression of genes related to antioxidant regulation and lipid metabolism through downstream genes, thereby affecting antioxidant capacity and lipid levels (Jiang et al., 2016a, 2016b; Shay et al., 2012; Liu and Sabatini, 2020). In related studies, increased levels of substituting fish meal with a plant protein source significantly reduced antioxidant enzyme activities and body lipid levels in animals, which was detrimental to health and growth of the animals (Liu et al., 2020; Xie et al., 2022; Zhang et al., 2020). To the best of our knowledge, the effect of CPC substitution on gene expression of antioxidant regulation and lipid metabolism has not been explored. In particular, there is no report on the use of CPC in Chinese mitten crab diets in the literature. The purpose of present study was to evaluate the feasibility of CPC in crab diet in terms of compositional characteristics, digestibility and utilization. The effects of fish meal substitution by CPC on growth performance, feed utilization, free amino acid profile and expression of genes related to antioxidant regulation, protein intake and nutrient metabolism of crabs were also analyzed to provide information for the rational use of CPC in crab diet.

2. Materials and methods

2.1. Ethics statement

The South China Normal University's Animal Care and Use Committee approved this study and the relevant guidelines were strictly followed in the conduct of this study (approval reference number SCNU-SLS-2023-040).

2.2. Test ingredients

The three protein sources tested in this study were Peruvian fish meal, soybean meal (Yihai Kerry Arawana Holdings Co, Ltd.) and CPC (Xinjiang Jinlan Plant Protein Co., Ltd.). Nutrient composition of protein sources is provided in Table 1.

2.3. Digestibility trial – experimental design and diet preparation

The formulation of the experimental diets is listed in Table 2. The reference diet was formulated using fish meal, soybean meal, peanut meal and krill meal as the main protein sources, fish oil and soybean oil as the main lipid sources, and wheat flour as the main carbohydrate source. Each test ingredient was combined in a 3:7 (wt:wt) ratio with the reference diet. As an inner digestibility marker, yttrium oxide (Y_2O_3; Shanghai Macklin Biochemical Co., Ltd.), was added to the reference diet at 0.1%.

All ingredients were screened through a 100-mesh sieve, weighed, mixed thoroughly, added with distilled water, and then pelleted into 2.0-mm diameter size by a double screw extruder (F-26, South China University of Technology) and a granulator (G-250, South China University of Technology). The pellets were cooked in a blast drier (DKN812C, Chongqing Yamato Technology Co., Ltd.) at 90 °C for 1.5 h. After drying, the pellets were store at −20 °C until use.

2.4. Digestibility trial – crab rearing and experimental conditions

Feeding experiments were performed in an indoor recirculating aquaculture system at Biology Park of South China Normal University. The water temperature, dissolved oxygen and pH were 27.0 ± 1.5 °C, >6.0 mg/L and 7.7 to 8.3, respectively. Nitrite

Table 1
Proximate composition and amino acid profile (%, based on dry weight) of the tested ingredients.

Item	Fish meal	Soybean meal	Cottonseed protein concentrate
Moisture	8.39	10.39	6.88
Crude protein	69.62	53.52	65.95
Crude lipid	11.01	1.41	0.68
Crude ash	17.06	7.46	8.00
EAA			
Arginine	3.79	3.81	8.26
Histidine	2.21	1.48	1.95
Isoleucine	2.44	2.30	1.81
Leucine	4.91	4.11	3.65
Lysine	5.31	3.27	2.70
Methionine	1.87	0.75	0.92
Phenylalanine	2.71	2.74	3.60
Threonine	2.92	2.14	2.07
Valine	3.07	2.46	2.70
ΣEAA	29.25	23.06	27.65
NEAA			
Alanine	4.39	2.43	2.47
Cysteine	0.62	0.72	1.08
Aspartic acid	6.32	6.20	6.15
Glutamic acid	8.77	9.74	13.39
Glycine	4.10	2.29	2.62
Serine	2.75	2.79	2.91
Proline	2.78	2.69	2.40
Tyrosine	1.98	1.98	2.01
ΣNEAA	31.70	28.84	33.02
ΣTAA	60.94	51.90	60.67

EAA = essential amino acids; NEAA = non-essential amino acids; TAA = total amino acids.

Table 2
Ingredient composition of the reference diet and test diet (%).

Ingredient	Reference diet	Test diet
Fish meal	28.00	19.60
Soybean meal	18.00	12.60
Krill meal	5.00	3.50
Peanut meal	8.00	5.60
Tested ingredients	0.00	30.00
Fish oil	2.00	1.40
Soybean oil	0.50	0.35
Phospholipid	2.00	1.40
Cholesterol	0.30	0.21
Wheat flour	28.00	19.60
$Ca(H_2PO_4)_2$	2.00	1.40
Choline chloride (60%)	0.10	0.07
Vitamin and mineral mixture[1]	1.00	0.70
Cellulose	5.00	3.50
Y_2O_3	0.10	0.07

[1] Vitamin and mineral mixture (IU or milligram per kilogram of mixture): vitamin A, ≥250,000 IU; vitamin D, 35,000 to 200,000 IU; vitamin E, ≥4000 mg; vitamin K_3, ≥1100 mg; vitamin B_1, ≥820 mg; vitamin B_2, ≥800 mg; vitamin B_6, ≥2500 mg; vitamin B_{12}, ≥5 mg; vitamin C, ≥12,000 mg; D-calcium pantothenate, ≥2500 mg; nicotinamide, ≥6000 mg; folic acid ≥500 mg; D-biotin, ≥10 mg; inositol, ≥3000 mg; magnesium, ≥5000 mg; zinc, 2360 to 15,000 mg; manganese, 630 to 15,000 mg; copper, 170 to 2500 mg; iron, 4500 to 75,000 mg; cobalt, 130 to 200 mg; iodine, 80 to 2000 mg; selenium, 26 to 50 mg; moisture, ≤10% (Guangdong Hinter Biotechnology Group Co., Ltd).

concentration was below 0.1 mg/L, and ammonia nitrogen concentration was below 0.2 mg/L.

Chinese mitten crabs were purchased from Chongming Breeding Base of Shanghai Ocean University and fed with commercial diet (Zhejiang Aohua Feed Co., LTD., crude protein content of 41%) for 2 weeks during acclimatization. At the beginning of experiment, 120 healthy crabs with an initial mean weight of 21.72 ± 0.33 g were selected and randomly divided into 12 fiberglass tanks (250 L, 3 tanks/diet, 10 crabs/tank). To reduce fighting, each tank was given 3 black arched tiles and 15 plastic pipes (5 pipes per bundle) as shelters. Crabs were fed twice daily at 07:30 and 16:30 during the culture experiment, and the residual diet and feces were collected 1.5 h after feeding. The culture experiment was conducted by collecting feces from the third week until sufficient fecal samples were available, lasting a total of 6 weeks.

2.5. Digestibility trial — feces sample collection and analysis

The fecal samples were gently rinsed with distilled water to remove debris. Daily collected feces were dried and stored at −20 °C for analysis of nutrients and yttrium.

2.6. Utilization trial — experimental design and diet preparation

The formulation and proximate composition of the experimental diets are listed in Table 3. Six isonitrogenous (44% crude protein) and isolipidic (8.5% crude lipid) diets were formulated using fish meal, soybean meal, rapeseed meal and krill meal as the main protein sources, fish oil and soybean oil as the main lipid sources, and wheat flour as the main carbohydrate source. The control group (FM) contained 35% fish meal, and five other diets (CPC15, CPC30, CPC45, CPC60 and CPC75 diets) were produced by replacing 15%, 30%, 45%, 60%, and 75% of fish meal with CPC, respectively. The final CPC inclusion levels were 0%, 5.90%, 11.70%, 17.60%, 23.50% and 29.40%, respectively. At each replacement level, Met and Lys were supplemented to maintain the consistency of the experimental diets. The amino acid composition of experimental diets is shown in Table 4. All ingredient handling and feed processing were as shown at Section 2.3, and the feed diameter was 1.0 mm.

2.7. Utilization trial — crab rearing and experimental conditions

Breeding equipment and conditions were as per Section 2.4. Chinese mitten crabs were purchased from Chongming Breeding Base of Shanghai Ocean University and fed with commercial diet (Zhejiang Aohua Feed Co., Ltd., crude protein content of 41%) for 2 weeks during acclimatization. At the beginning of experiment, 630 healthy crabs with an initial mean weight of 1.68 ± 0.00 g were selected and distributed randomly into 18 fiberglass tanks (250 L, 3 tanks/diet, 35 crabs/tank). Five black arched tiles and 25 plastic pipes (5 pipes per bundle) were placed in each tank as a shelter to mitigate the fighting behavior. Crabs were fed 3 times daily at 07:30, 16:30 and 21:30 during the culture experiment, and the residual diet and feces were cleaned 1.5 h after feeding. The culture experiment lasted for 9 weeks.

2.8. Utilization trial — sample collection and analysis

At the end of the 9-week trial, crabs in each tank were counted and weighed to assess growth performance, feed utilization, and survival. Eight samples were randomly selected from each tank, and their hemolymph was extracted with 1.0 mL syringes, transferred into 1.5-mL Eppendorf (EP) tube, placed at 4 °C for 5 h and centrifuged at 1900 × *g* for 15 min. The upper serum was separated and frozen at −80 °C for subsequent biochemical index analysis. The hepatopancreas, intestine and muscle of each crab were then removed and quickly placed into pre-labeled and pre-cooled 1.5-mL EP tubes, which was frozen in liquid nitrogen and stored at −80 °C until further analysis of gene expression and amino acid composition. Six crabs were randomly chosen from each tank and put in a marked sealed bags and stored at −20 °C in order to assess whole-body proximate composition.

Table 3
Ingredients and proximate composition of the experimental diets (%).

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75
Ingredients						
Fish meal	35.00	29.75	24.50	19.25	14.00	8.75
Cottonseed protein concentrate	0.00	5.90	11.70	17.60	23.50	29.40
Soybean meal	14.00	14.00	14.00	14.00	14.00	14.00
Rapeseed meal	6.00	6.00	6.00	6.00	6.00	6.00
Krill meal	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil	0.00	0.40	0.80	1.20	1.60	2.00
Soybean oil	2.50	2.50	2.50	2.50	2.50	2.50
Phospholipid	2.00	2.00	2.00	2.00	2.00	2.00
Cholesterol	0.30	0.30	0.30	0.30	0.30	0.30
Wheat flour	25.99	24.76	23.62	22.39	21.15	19.92
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride (60%)	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin and mineral mixture[1]	1.00	1.00	1.00	1.00	1.00	1.00
Cellulose	6.00	6.00	6.00	6.00	6.00	6.00
Carboxymethylcellulose sodium	0.01	0.01	0.01	0.01	0.01	0.01
Y_2O_3	0.10	0.10	0.10	0.10	0.10	0.10
Lysine	0.00	0.13	0.26	0.39	0.52	0.65
Methionine	0.00	0.05	0.11	0.16	0.22	0.27
Proximate composition (based on dry weight)						
Moisture	10.29	10.06	9.88	9.53	9.62	9.85
Crude protein	44.08	44.40	44.05	44.06	44.22	44.39
Crude lipid	8.54	8.72	8.48	8.43	8.38	8.25
Crude ash	10.08	9.90	9.55	9.05	8.76	8.36

[1] Vitamin and mineral mixture (IU or milligram per kilogram of mixture): vitamin A, ≥250,000 IU; vitamin D, 35,000 to 200,000 IU; vitamin E, ≥4000 mg; vitamin K_3, ≥1100 mg; vitamin B_1, ≥820 mg; vitamin B_2, ≥800 mg; vitamin B_6, ≥2500 mg; vitamin B_{12}, ≥5 mg; vitamin C, ≥12,000 mg; D-calcium pantothenate, ≥2500 mg; nicotinamide, ≥6000 mg; folic acid ≥500 mg; D-biotin, ≥10 mg; inositol, ≥3000 mg; magnesium, ≥5000 mg; zinc, 2360 to 15,000 mg; manganese, 630 to 15,000 mg; copper, 170 to 2500 mg; iron, 4500 to 75,000 mg; cobalt, 130 to 200 mg; iodine, 80 to 2000 mg; selenium, 26 to 50 mg; moisture, ≤10% (Guangdong Hinter Biotechnology Group Co., Ltd).

Table 4
Amino acids of the experimental diets (%, based on dry weight).

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75
EAA						
Arginine	2.35	2.61	2.80	3.03	3.24	3.49
Histidine	0.99	1.01	1.01	1.02	1.03	1.06
Isoleucine	1.81	1.67	1.59	1.51	1.45	1.38
Leucine	3.18	3.08	2.95	2.87	2.77	2.68
Lysine	2.17	2.14	2.13	2.09	2.12	2.07
Methionine	0.99	0.98	1.00	0.98	0.98	0.98
Phenylalanine	1.90	1.93	1.94	2.00	2.04	2.07
Threonine	1.84	1.77	1.73	1.66	1.60	1.54
Valine	2.24	2.13	2.06	1.99	1.93	1.88
ΣEAA	17.46	17.32	17.22	17.16	17.16	17.16
NEAA						
Alanine	2.46	2.38	2.25	2.17	2.04	1.93
Cysteine	0.51	0.55	0.57	0.58	0.61	0.63
Aspartic acid	4.12	4.12	4.05	4.03	4.02	3.98
Glutamic acid	6.88	7.18	7.37	7.65	7.93	8.15
Glycine	2.30	2.24	2.15	2.10	2.01	1.94
Serine	1.89	1.90	1.93	1.94	1.94	1.96
Proline	2.06	2.05	2.01	2.00	1.96	1.93
Tyrosine	1.24	1.29	1.23	1.25	1.24	1.21
ΣNEAA	21.45	21.71	21.58	21.71	21.74	21.73
ΣTAA	38.91	39.02	38.80	38.97	38.90	38.88

EAA = essential amino acids; NEAA = non-essential amino acids; TAA = total amino acids.

2.9. Digestibility calculation

ADC of dry matter, crude protein and amino acids of the reference diet and test diets were calculated using the formula as follows:

ADC of dry matter (%) = 100 − (100 × Y_2O_3 in diet/Y_2O_3 in feces),

ADC of nutrient (%) = 100 − [100 × (Y_2O_3 in diet/Y_2O_3 in feces) × (nutrient in feces/nutrient in diet)].

ADC of dry matter was calculated using dry matter instead of nutrient data.

ADC of the test ingredients were calculated based on the digestibility of the reference diet and experiment diets using the equation (Bureau and Hua, 2006):

$$ADC_i = ADC_{td} + [(ADC_{td} - ADC_{rd}) \times (I_{rd} \times N_{rd})/(I_i \times N_i)],$$

where ADC_i = apparent digestibility coefficient of dry matter or nutrient of the ingredients, ADC_{td} = apparent digestibility

coefficient of the test diet, ADC_{rd} = apparent digestibility coefficient of the reference diet, I_{rd} = % of the reference diet in the mash, I_i = % of the test ingredient in the mash, N_{rd} = % of nutrient in the reference diet mash (as fed), and N_i = % of nutrient in the test ingredient (as fed).

2.10. Growth performance and feed utilization

The following variables were calculated:

WG (%) = 100 × (final body weight − initial body weight)/initial body weight,

Specific growth rate (SGR, %/day) = 100 × [Ln (final body weight) − Ln (initial body weight)]/total experimental days,

Survival rate (SR, %) = 100 × (final number of crab/initial number of crab),

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

FI (g/crab) = total FI/final number of crabs,

Feed conversion ratio (FCR) = FI/(final body weight − initial body weight),

Protein efficiency ratio (PER) = (final body weight − initial body weight)/protein intake,

Protein retention (PR, %) = 100 × [(final body weight × final body protein content) − (initial body weight × initial body protein content)]/protein intake.

2.11. Proximate composition analysis

Proximate composition of ingredients, experimental diets, and whole-body samples was analyzed in triplicate according to standard methods (AOAC, 2005). Moisture was determined by oven drying at 105 °C in a blast dryer (DKN812C, Chongqing Yamato Technology Co., Ltd.) to constant weight. Crude protein content was estimated using the Dumas nitrogen determination apparatus (Dumatherm DT NPRO, Gerhart Analytical Systems, Germany). Crude lipid was determined using the Soxhlet extraction with petroleum ether (boiling range 60 to 90 °C). Crude ash was analyzed after the sample was fully carbonized and placed in muffle furnace (FO610C, Yamato Scientific Co., Ltd., Tokyo, Japan) after burning at 550 °C for 6 h. The amino acid composition was measured by Guangzhou Chengyi Aquatic Technology Co., Ltd., according to GB/T 18246-2019 (China National Standard, 2019), using an oxidative hydrolysis method with an Automatic Amino Acid Analyzer (Biochrom 30^+, Biochrom Ltd., Britain).

2.12. Serum biochemical indices analyses

Serum total protein (TP) was measured using the bicinchoninic acid (BCA) protein assay kit (P0011, Beyotime, Nantong, China) according to the manufacturer's instructions. Triglyceride (TG), total cholesterol (T-CHO), glucose concentrations, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using the commercial assay kits (A110-1, A111-1, F006-1, C009-2, C010-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.13. Hepatopancreas antioxidant enzymes activity assays

The hepatopancreas samples were precisely weighed and mixed with PBS buffer in accordance with the weight (g) to volume (mL) ratio of 1:9. Then it was homogenized in a refrigerated grinder and centrifuged at 13,700 × *g* for 15 min at 4 °C. An aliquot of the supernatant was taken for further analysis. The activities of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) were determined using the commercial assay kits (A001-3 and A015-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.14. Free amino acids determination

Approximately 100 mg of freeze-dried tissue sample was placed into 2.0-mL EP tube and 1.2 mL 10% sulfosalicylic acid was added, and the mixture was thoroughly shaken and centrifuged at 13,700 × *g* for 15 min at 4 °C. The supernatant was filtered by 0.22-μm membrane and transferred into 2.0-mL glass thread screw neck vials with 9-mm screw cap that contained a Red Silicone/Ivory PTFE (Thermo scientific, America). The composition and concentration of free amino acids in tissues were determined by High-speed Amino Acid Analyzer (L-8900, Hitachi High-Technologies Co., Tokyo, Japan).

2.15. Gene expression by quantitative real-time PCR (q-PCR)

The expression levels of genes related to antioxidant, protein metabolism and lipid metabolism were detected using q-PCR. Total RNA was isolated from the intestine and hepatopancreas with Trizol (9109, Takara, Japan). The purity of RNA was determined on a Nano Drop 2000 spectrophotometer (Thermo, USA). The following analysis only utilized RNA samples having the A260/A280 ratio between 1.8 and 2.0. And RNA integrity was verified using a 1.0% agarose gel. For each reverse transcription reaction, 1000 ng of RNA samples was treated with 5× gDNA digester Mix (Yeasen, Shanghai, China) to avoid genomic DNA amplification, and then reverse-transcribed into cDNA using 4× Hifair III SuperMix plus (Yeasen, Shanghai, China). The sequences of the primers are shown in Table 5.

Comparative gene mRNA quantification was employed. The q-PCR was conducted with an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) in a 20 μL reaction mixture including 0.5 μL PCR forward primer (10 μm), 0.5 μL PCR reverse primer (10 μm), 2.0 μL cDNA solution, 10 μL SYBR Green Master Mix, and 7.0 μL dH_2O. The reaction conditions were as follows: 95 °C for 5 min followed by 40 cycles consisting of 60 °C for 30 s and 95 °C for 15 s. The correlation coefficients (R^2) of all standard curves were >0.98 and amplification efficiencies were between 90% and 110%. The expression levels of genes were calculated using $2^{-\triangle\triangle CT}$ method, and the normalization response was carried out with the ubiquitin/ribosomal S27 fusion protein (*s27*) gene as a reference.

2.16. Statistical analysis

Data are expressed as means and pooled SEM. One-way ANOVA was used to analyze the data. The differences with *P*-values < 0.05 were deemed significant in all analyses using the SPSS 25.0. Orthogonal polynomial contrasts were used in a subsequent trend analysis to assess if the significant effect was linear and/or quadratic. The correlations of amino acid changes in diet and tissues were estimated by Pearson's correlation.

Table 5
The sequence of the primers used for real-time quantitative PCR.

Gene	Primer sequences (5′ to 3′)	Genbank accession no.	Function	Length, bp
cu-zn sod	F-ATGAGTAAGACCTTTGCCTA	XM_050882595	Antioxidation	20
	R-TCCGTCAGTCCATAGATAAC			20
gpx	F-GACTACACCCCAGTGTGCACCA	XM_050864461	Antioxidation	22
	R-TGATCCAGCCATTGTGATCCTC			22
cnccc	F-GCATCCTTCTGGTACCTCGTT	XM_050837162	Antioxidation	21
	R-CACTGCTTTGGCTCATCCTTG			21
keap1	F-CTTCATGTACACTGGCGAGA	XM_050879111	Antioxidation	20
	R-GTACAGCAAGCGTCAATCACA			20
pept1	F-ACAGAGGAGCAGCACCAGG	XM_050870767	Peptide transport	19
	R-CATTGCCGACGATGAAGAA			19
slc7a5	F-GTCCTTCGCGCTGTGGTTGA	XM_050876303	Amino acids transport	20
	R-GGGCCGTGTGCACCTTGATA			20
erk	F-CTGTGAGTAGCAGGGCATA	XM_050877376	TOR pathway	19
	R-GGTTCAGGCAACATCAAG			18
rheb	F-ATGCTCCTTGGCACTTGTCTC	XM_050879238	TOR pathway	21
	R-TCCTGTGGTGTTGGTGGGTA			20
raptor	F-ACGAGCACTGGAACTGTTAGGA	XM_050875410	TOR pathway	22
	R-AGTGGACTGAAGCAGCCGTA			20
tor	F-GCAGCCCCAAGGAGATGAAA	XM_050855996	TOR pathway	20
	R-ACAGTCGAAACGCCCTCATC			20
s6k1	F-GCACCAGGCTTATTCGACCT	XM_050854928	TOR pathway	20
	R-GGGTTGACTGTGCTGTCTGA			21
4ebp	F-CACGAAACCGACTACTGC	XM_050856547	TOR pathway	18
	R-CCAAGACCTGATGATGAAC			19
eif4e	F-CAAGCAGTAGTCCCTCACAAA	XM_050881025	TOR pathway	21
	R-GTGGCCTTACACACAGTAGTC			21
srebp1	F-TCTTCACACCCTCTGGACGC	XM_050880187	Lipid metabolism	20
	R-CCAAGGTTGTAATGGCACGC			20
△9 fad	F-TGGCACAACTACCACCACGTCT	XM_050880689	Lipid metabolism	22
	R-TCCTCTTCTCGATCATCTCCGG			22
cpt1	F-CATCTGGACACCCACCTCCA	XM_050841771	Lipid metabolism	20
	R-ATCTCCTCACCCGGCACTCT			20
caat	F-CATCAAGAGCCAGGAGCCCA	XM_050835164	Lipid metabolism	20
	R-CTTCAACAGCAGCCCGCAAA			20
s27	F-GGTCGATGACAATGGCAAGA	XM_050861302	Housekeeping gene	20
	R-CCACAGTACTGGCGGTCAAA			20

F = forward; R = reverse; *cu-zn sod* = copper-zinc superoxide dismutase; *gpx* = glutathione peroxidase; *cncc* = cap 'n' collar isoform C; *keap1* = Kelch-like ECH-associated protein 1; *pept1* = peptide transporter 1; *slc7a5* = solute carrier family 7 member 5; *erk* = extracellular regulated protein kinases; *rheb* = Ras homolog enriched in brain; *raptor* = regulator-associated protein of TOR; *tor* = rapamycin target protein; *s6k1* = ribosomal protein S6 kinase 1; *4ebp* = eIF4E binding protein; *eif4e* = eukaryotic translation initiation factor 4E; *srebp1* = sterol regulatory element-binding protein 1; *Δ9 fad* = Δ9 fatty acyl desaturase; *cpt1* = carnitine palmitoyltransferase 1; *caat* = carnitine acetyl-transferase; *s27* = ubiquitin/ribosomal S27 fusion protein.

3. Results

3.1. Nutritional composition of test ingredients

As shown in Table 1, the crude protein concentration of CPC was 65.95%, which was close to fish meal (69.62%) and substantially higher than soybean meal (53.52%). The crude lipid concentration of fish meal (11.01%) was higher, and that of soybean meal (1.41%) and CPC (0.68%) was lower. Compared to soybean meal (7.46%) and CPC (8.00%), fish meal had a higher crude ash concentration (17.06%). Among the amino acids, the contents of Ile (1.81%), Leu (3.65%), Lys (2.70%), Met (0.92%) and Thr (2.07%) of CPC were lower than those of fish meal (Ile 2.44%, Leu 4.91%, Lys 5.31%, Met 1.87%, Thr 2.92%). However, the contents of Arg (8.26%) and Phe (3.60%) of CPC were higher than those of fish meal (Arg 3.79%, Phe 2.71%). The essential amino acid (EAA, 27.65%) contents of CPC were lower than that of fish meal (EAA, 29.25%), and the total amino acid (TAA, 60.67%) content was close to that of fish meal (TAA, 60.94%).

3.2. Apparent digestibility of nutrients in test ingredients by E. sinensis

Table 6 shows the ADC of the dry matter, crude protein and amino acids of test ingredients. There was no discernible difference between the dry matter ADC of three protein sources, which varied from 70.68% to 73.50% for crabs ($P > 0.05$). The protein ADC of fish meal (87.21%) and CPC (90.42%) was significantly higher than that of soybean meal (83.16%) ($P < 0.05$).

Among the three protein sources, the ADC of Leu, Met, Thr and Ala of soybean meal and CPC were significantly lower than those of fish meal ($P < 0.05$). The ADC values of Ile and Lys in CPC were significantly lower, while Phe, Cys and Glu were significantly higher than those in fish meal and soybean meal ($P < 0.05$). The ADC of the remaining amino acids, EAA, and TAA were not significantly different among three protein sources ($P > 0.05$).

3.3. Growth performance and feed utilization

As shown in Table 7, when the basal diet contained 35% fish meal, of which 75% was replaced by CPC, crabs showed significantly lower final body weight, WG, SGR, PER and PR, and significantly higher FCR ($P < 0.05$). When CPC replaced 0% to 60% of fish meal, final body weight, WG, SGR, PER, PR and FCR of crabs were not significantly affected. The SR, FI, and HSI of crabs under various treatments did not differ significantly from one another ($P > 0.05$). Fish meal replacement with CPC had significant linear and quadratic impacts in FCR and PR, but only a quadratic effect in PER ($P < 0.05$). According to the quadratic regression analysis of FCR and PER with dietary CPC content, the optimal additions of CPC were 12.62% and 13.80%, respectively (Fig. 1), which corresponded to

Table 6
Apparent digestibility coefficients (%) of dry matter, crude protein, and amino acids of the test ingredients.

Item	Fish meal	Soybean meal	Cottonseed protein concentrate	SEM	*P*-value
Proximate composition					
Dry matter	73.50	70.68	72.62	1.137	0.650
Crude protein	87.21^{b}	83.16^{a}	90.42^{b}	1.164	0.006
Amino acids					
Arginine	93.87^{ab}	93.27^{a}	95.16^{b}	0.332	0.029
Histidine	92.24	89.30	91.54	0.632	0.131
Isoleucine	91.54^{b}	89.92^{b}	86.05^{a}	0.987	0.033
Leucine	93.55^{b}	90.30^{a}	89.08^{a}	0.800	0.028
Lysine	94.47^{c}	89.92^{b}	82.47^{a}	1.801	<0.001
Methionine	92.76^{b}	87.87^{a}	86.18^{a}	1.171	0.025
Phenylalanine	87.19^{a}	88.84^{a}	91.74^{b}	0.776	0.019
Threonine	90.36^{b}	87.93^{a}	87.28^{a}	0.583	0.044
Valine	90.97	88.41	88.03	0.633	0.103
Alanine	90.81^{b}	86.66^{a}	86.32^{a}	0.829	0.014
Cysteine	85.21^{a}	81.91^{a}	90.89^{b}	1.401	0.002
Aspartic acid	89.99	91.43	91.67	0.396	0.172
Glutamic acid	93.11^{a}	93.56^{a}	94.94^{b}	0.332	0.031
Glycine	85.55	84.89	86.43	1.087	0.879
Serine	89.09	89.70	91.08	0.410	0.119
Proline	89.18	89.63	90.16	0.354	0.588
Tyrosine	94.51	94.29	92.92	0.616	0.583
EAA	92.26	90.13	90.21	0.502	0.140
TAA	91.41	90.61	91.26	0.320	0.617

EAA = essential amino acid; TAA = total amino acid.
Values are mean of 3 replicates. $^{a-c}$Values not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$). *P*-value for ANOVA.

Table 7
Growth performance and feed utilization of *Eriocheir sinensis* fed with different experimental diets for 9 weeks.

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75	SEM	*P*-value		
								ANOVA	Linear	Quadratic
FBW, g	6.14^{b}	5.87^{b}	5.99^{b}	5.75^{b}	6.24^{b}	5.16^{a}	0.103	0.009	0.014	0.117
WG, %	265.07^{b}	250.58^{b}	256.90^{b}	243.70^{b}	273.43^{b}	207.45^{a}	6.219	0.010	0.019	0.109
SGR, %/d	2.05^{b}	1.99^{b}	2.02^{b}	1.96^{b}	2.09^{b}	1.78^{a}	0.029	0.010	0.016	0.095
SR, %	78.10	68.57	82.86	78.10	74.29	78.10	2.069	0.532	0.780	0.906
HSI, %	8.45	8.54	9.33	8.65	8.86	8.46	0.104	0.084	0.841	0.029
FI, g/crab	5.59	5.13	5.19	4.85	5.36	4.85	0.100	0.222	0.100	0.482
FCR	1.25^{a}	1.22^{a}	1.20^{a}	1.19^{a}	1.18^{a}	1.39^{b}	0.020	0.002	0.040	<0.001
PER	1.81^{b}	1.85^{b}	1.89^{b}	1.90^{b}	1.92^{b}	1.62^{a}	0.028	0.003	0.063	0.001
PR, %	27.70^{b}	25.86^{ab}	27.23^{b}	27.16^{b}	27.34^{b}	21.91^{a}	0.618	0.026	0.022	0.046

FBW = final body weight; WG = weight gain; SGR = specific growth rate; SR = survival rate; HSI = hepatosomatic index; FI = feed intake; FCR = feed conversion ratio; PER = protein efficiency ratio; PR = protein retention.
Values are mean of 3 replicates. ^{a,b}Values not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

replacing 32.36% and 35.38% of the fish meal of the basal diet (containing 35% fish meal), respectively.

3.4. Proximate composition of whole crab

No significant effect of dietary CPC levels on the moisture, crude protein, lipid, and ash contents of whole crabs was observed (Table 8).

3.5. Biochemical indexes of serum

Table 9 shows the results of substituting fish meal by CPC on serum biochemical index of crabs. The concentration of T-CHO was linearly and quadratically affected by different dietary CPC levels ($P < 0.05$), while only quadratic effect was detected in the concentration of TG ($P < 0.05$). A significant positive linear trend existed between the activity of ALT and dietary CPC level ($P < 0.05$). Crabs fed the CPC15, CPC30, and CPC45 diets had higher serum TG concentrations than crabs fed other diets, and crabs fed the CPC30 and CPC45 diets had considerably higher serum T-CHO concentrations ($P < 0.05$). There were no discernible variations in the levels of TP, glucose, or AST activity across all treatments ($P > 0.05$).

3.6. Antioxidation capacity in hepatopancreas

As shown in Fig. 2, crabs fed the CPC75 diet had lower activity of SOD and lower level of gene expression of glutathione peroxidase (*gpx*) than those fed the other diets ($P < 0.05$). Copper-zinc superoxide dismutase (*cu-zn sod*), and cap 'n' collar isoform C (*cncc*) mRNA expression levels were significantly greater in CPC15 group crabs, whereas Kelch-like ECH-associated protein 1 (*keap1*) mRNA expression levels were significantly lower ($P < 0.05$). Fish meal substituted with CPC has no impact on the T-AOC value in the hepatopancreas ($P > 0.05$).

3.7. Free amino acid compositions of muscle and hepatopancreas

Free amino acids contents in muscle are shown in Table 10. The contents of Ile, Leu, Lys, Met, Thr and the total content of free EAA were the lowest in CPC75 group ($P < 0.05$). When compared to crabs fed other group diets, those fed the FM diet had muscles with a larger amount of Asp ($P < 0.05$). As the levels of CPC in diets increased, the content of Ala in crab muscles showed a tendency to increase after decrease, with higher levels in the CPC30, CPC45 and CPC60 groups ($P < 0.05$). The total amount of free non-essential

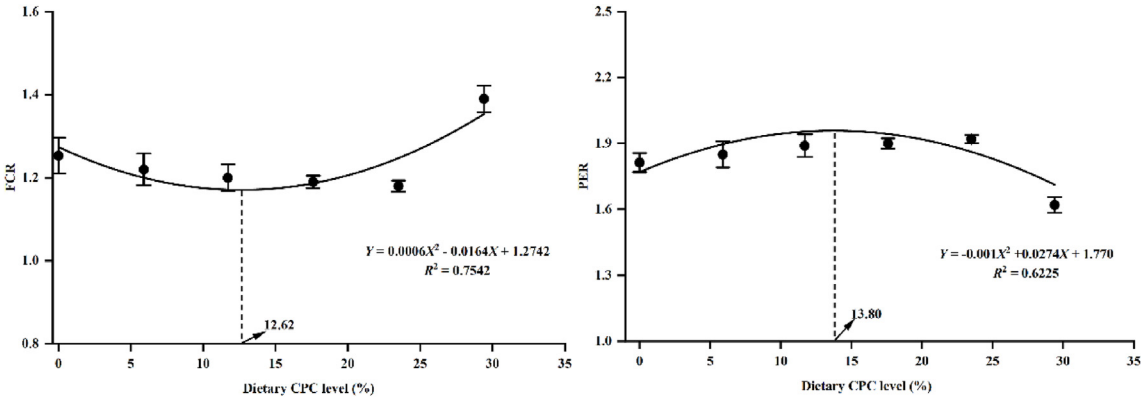

Fig. 1. Quadratic regression analysis of feed conversion ratio (FCR) and protein efficiency ratio (PER) in *Eriocheir sinensis* fed with different CPC contents.

Table 8
Proximate composition in whole body of *Eriocheir sinensis* fed with different experimental diets for 9 weeks (%, based on wet weight).

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75	SEM	*P*-value		
								ANOVA	Linear	Quadratic
Moisture	63.44	65.63	64.40	63.88	63.99	65.66	0.397	0.512	0.507	0.769
Crude protein	14.44	13.50	13.79	13.93	13.67	13.06	0.167	0.284	0.075	0.886
Crude lipid	3.63	3.22	3.36	3.13	3.95	3.84	0.127	0.345	0.264	0.149
Crude ash	12.68	12.36	12.75	13.22	12.65	12.34	0.133	0.487	0.902	0.233

Values are mean of 3 replicates.

Table 9
Serum biochemical indices of *Eriocheir sinensis* fed with different experimental diets for 9 weeks.

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75	SEM	*P*-value		
								ANOVA	Linear	Quadratic
TP, g/L	62.87	59.51	66.94	68.42	64.35	64.16	1.045	0.173	0.263	0.176
TG, mmol/L	0.14^{a}	0.22^{c}	0.19^{bc}	0.22^{c}	0.17^{ab}	0.16^{ab}	0.008	0.001	0.796	<0.001
T-CHO, mmol/L	0.42^{a}	0.41^{a}	0.64^{b}	0.65^{b}	0.57^{ab}	0.58^{ab}	0.030	0.027	0.014	0.045
Glucose, mmol/L	2.37	2.36	2.07	2.28	2.17	2.08	0.059	0.557	0.164	0.850
ALT, U/L	67.86^{a}	59.57^{a}	71.88^{ab}	90.24^{cd}	82.63^{bc}	100.08^{d}	3.601	<0.001	<0.001	0.200
AST, U/L	8.10	8.35	7.69	8.90	8.23	9.43	0.290	0.641	0.255	0.501

TP = total protein; TG = triglyceride; T-CHO = total cholesterol; ALT = alanine aminotransferase; AST = aspartate aminotransferase.
Values are mean of 3 replicates. $^{a-d}$Values not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

amino acids (NEAA) and delicious amino acids (DAA) in muscle has no change when fish meal is substituted with CPC ($P > 0.05$).

Fish meal was substituted with CPC without affecting hepatopancreatic Asp and Pro (Table 11). Except Asp and Pro, other free amino acids of hepatopancreas were significantly affected by CPC and gradually decreased as the level of dietary CPC increased ($P < 0.05$). DAA also decreased as the level of dietary CPC increased ($P < 0.05$). The free amino acids concentrations of hepatopancreas and the level of CPC substituted fish meal linear variation were shown in Table 12. The findings showed that the free amino acids content, except for Arg, Asp and Pro, were negatively correlated with the CPC substitution level.

3.8. Correlation between changes in amino acid composition of the diet and tissues

The Pearson correlation coefficient between the dietary amino acid content and the tissue free amino acid content is shown in Fig. 3. Muscle Ile, Val and Gly levels were positively correlated with dietary Ile, Val and Gly contents, while His was negatively correlated ($P < 0.05$). In hepatopancreas, the concentrations of 13 free amino acids showed a significant linear correlation with the corresponding amino acid contents in the diet. Among them, the changes of Ile, Leu, Thr, Val, Ala, Gly and Pro were positively correlated, while that of Arg, His, Phe, Cys, Glu and Ser were negatively correlated ($P < 0.05$).

3.9. Expression levels of genes related to protein transport and TOR signaling pathway in the intestine

The expression of peptide transporter 1 (*pept1*), solute carrier family 7 member 5 (*slc7a5*), *tor* and *s6k1* were negatively correlated with dietary CPC level, as shown in Fig. 4 ($P < 0.05$). Crabs in the CPC60 and CPC75 groups showed significantly higher levels of *4ebp* mRNA expression ($P < 0.05$).

3.10. Expression levels of genes related to TOR signaling pathway and lipid metabolism in hepatopancreas

As shown in Fig. 5A, the expressions of *tor*, *s6k1*, regulatory-associated protein of TOR (*raptor*) and *rheb* were the highest in CPC15 group, but as CPC progressively replaced fish meal, the

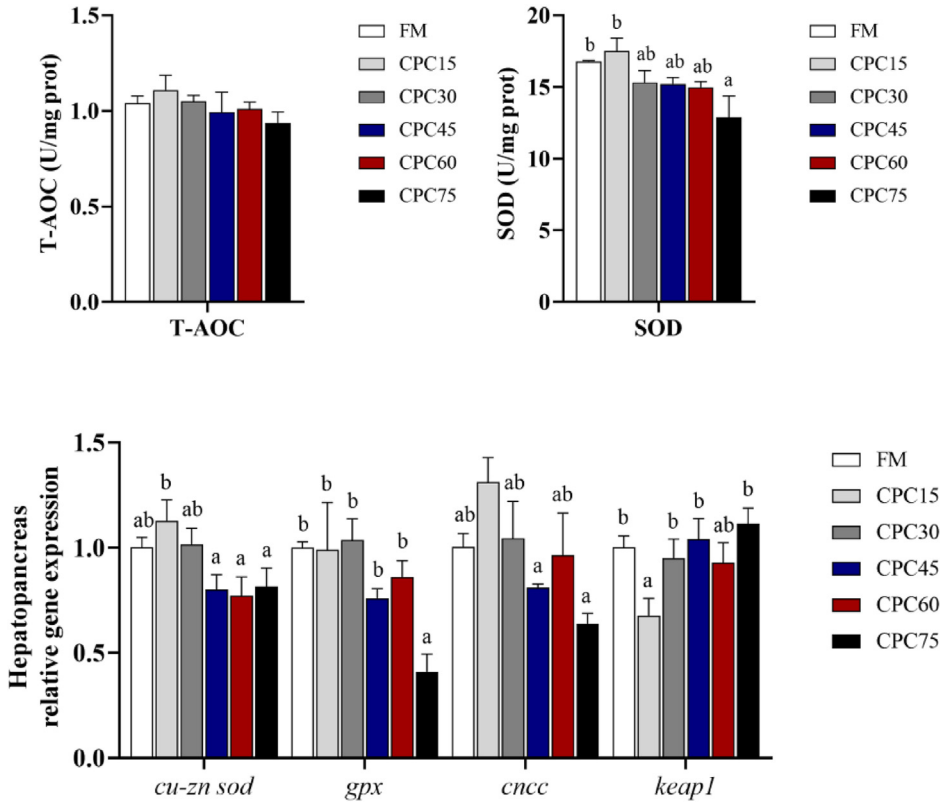

Fig. 2. The antioxidant enzyme activities and expression levels of genes related to antioxidant regulation in hepatopancreas of *Eriocheir sinensis* fed different experimental diets for 9 weeks. T-AOC = total antioxidant capacity; SOD = superoxide dismutase; *cu-zn sod* = copper-zinc superoxide dismutase; *gpx* = glutathione peroxidase; *cncc* = cap 'n' collar isoform C; *keap1* = Kelch-like ECH-associated protein 1. [a,b]Bars of the same gene not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

Table 10
Free amino acids composition in muscle of *Eriocheir sinensis* fed with different experimental diets for 9 weeks (mg/g, based on dry matter).

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75	SEM	*P*-value		
								ANOVA	Linear	Quadratic
EAA										
Arginine	20.17	19.52	20.09	21.78	21.08	20.59	0.178	0.111	0.033	0.937
Histidine	1.07	1.10	1.05	0.99	1.00	0.94	0.024	0.483	0.071	0.693
Isoleucine	0.57^{c}	0.44^{b}	0.46^{b}	0.41^{b}	0.43^{b}	0.28^{a}	0.022	<0.001	<0.001	0.747
Leucine	1.95^{b}	2.10^{b}	2.13^{b}	1.95^{b}	2.01^{b}	1.48^{a}	0.056	<0.001	<0.001	<0.001
Lysine	1.83^{bc}	1.73^{bc}	1.89^{c}	1.64^{b}	1.73^{bc}	1.21^{a}	0.058	<0.001	<0.001	0.001
Methionine	1.47^{b}	1.45^{b}	1.62^{b}	1.47^{b}	1.46^{b}	1.05^{a}	0.046	<0.001	<0.001	<0.001
Phenylalanine	0.55	0.54	0.61	0.51	0.51	0.49	0.016	0.321	0.135	0.353
Threonine	1.31^{b}	1.31^{b}	1.26^{b}	1.22^{b}	1.38^{b}	0.81^{a}	0.052	0.001	0.001	0.011
Valine	1.17	1.15	1.16	0.99	1.06	0.99	0.029	0.178	0.027	0.997
ΣEAA	30.10^{bc}	29.34^{b}	30.26^{bc}	29.95^{bc}	30.68^{c}	27.84^{a}	0.252	0.001	0.021	0.004
NEAA										
Alanine	6.71^{a}	7.03^{ab}	7.27^{b}	7.36^{b}	7.27^{b}	6.58^{a}	0.089	0.016	0.914	0.001
Cysteine	0.02	0.02	0.02	0.02	0.02	0.02	<0.001	0.375	0.484	0.092
Aspartic acid	1.30^{c}	0.52^{ab}	0.48^{a}	0.51^{ab}	0.61^{b}	0.56^{ab}	0.072	<0.001	<0.001	<0.001
Glutamic acid	2.40	2.12	2.12	2.08	2.48	2.23	0.055	0.167	0.846	0.138
Glycine	6.33	6.20	6.15	6.15	6.09	6.08	0.085	0.980	0.450	0.825
Serine	0.87^{c}	0.71^{abc}	0.82^{c}	0.57^{a}	0.77^{bc}	0.59^{ab}	0.030	0.024	0.026	0.505
Proline	15.74	15.57	15.59	16.10	16.33	15.10	0.163	0.338	0.893	0.231
Tyrosine	1.06	1.04	0.95	0.89	0.88	0.86	0.028	0.140	0.009	0.575
ΣDAA	16.74	15.87	16.01	16.10	16.45	15.45	0.166	0.301	0.176	0.969
ΣNEAA	34.43	33.21	33.41	33.68	34.45	32.01	0.289	0.115	0.135	0.504

EAA = essential amino acids; DAA = delicious amino acids (including alanine, aspartic acid, glutamic acid and glycine); NEAA = non-essential amino acids.
Values are mean of 3 replicates. [a–c]Values not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

expression levels of *tor* and *raptor* significantly down-regulated ($P < 0.05$). The gene expression of *eif4e* and extracellular regulated protein kinases (*erk*) decreased significantly as the level of dietary CPC increased ($P < 0.05$).

The expression of sterol regulatory element-binding protein 1 (*srebp1*), Δ9 fatty acyl desaturase (*Δ9 fad*) and carnitine palmitoyltransferase 1 (*cpt1*) were the highest in CPC15 group, and significantly decreased as fish meal substitution by CPC increased

Table 11
Free amino acids composition in hepatopancreas of *Eriocheir sinensis* fed with different experimental diets for 9 weeks (mg/g, based on dry matter).

Item	FM	CPC15	CPC30	CPC45	CPC60	CPC75	SEM	*P*-value		
								ANOVA	Linear	Quadratic
EAA										
Arginine	3.93^{c}	3.56^{abc}	3.50^{ab}	3.67^{bc}	3.31^{ab}	3.28^{a}	0.066	0.014	0.002	0.667
Histidine	0.58^{c}	0.45^{b}	0.46^{bc}	0.43^{b}	0.35^{ab}	0.28^{a}	0.026	0.003	<0.001	0.839
Isoleucine	0.89^{c}	0.68^{b}	0.71^{b}	0.63^{b}	0.56^{ab}	0.43^{a}	0.038	0.001	<0.001	0.906
Leucine	2.27^{d}	1.68^{bc}	1.85^{c}	1.69^{bc}	1.51^{b}	1.21^{a}	0.083	<0.001	<0.001	0.916
Lysine	2.84^{d}	2.14^{bc}	2.36^{c}	2.28^{bc}	2.04^{ab}	1.78^{a}	0.085	<0.001	<0.001	0.695
Methionine	0.88^{c}	0.65^{b}	0.72^{b}	0.66^{b}	0.59^{ab}	0.48^{a}	0.034	0.001	<0.001	0.856
Phenylalanine	1.59^{d}	1.15^{bc}	1.32^{c}	1.11^{bc}	1.05^{b}	0.80^{a}	0.063	<0.001	<0.001	0.964
Threonine	0.83^{d}	0.63^{c}	0.66^{c}	0.59^{bc}	0.49^{ab}	0.40^{a}	0.035	<0.001	<0.001	0.936
Valine	1.15^{d}	0.87^{bc}	0.97^{c}	0.88^{bc}	0.76^{ab}	0.66^{a}	0.041	<0.001	<0.001	0.957
ΣEAA	14.96^{d}	11.82^{bc}	12.56^{c}	11.94^{bc}	10.67^{b}	9.31^{a}	0.445	<0.001	<0.001	0.820
NEAA										
Alanine	1.60^{c}	1.45^{bc}	1.44^{bc}	1.37^{b}	1.15^{a}	1.08^{a}	0.047	<0.001	<0.001	0.358
Cysteine	0.17^{d}	0.12^{c}	0.13^{c}	0.10^{b}	0.09^{b}	0.07^{a}	0.008	<0.001	<0.001	0.265
Aspartic acid	0.29	0.29	0.25	0.25	0.24	0.32	0.011	0.211	0.880	0.040
Glutamic acid	2.03^{c}	1.86^{bc}	1.79^{bc}	1.60^{ab}	1.38^{a}	1.38^{a}	0.066	0.001	<0.001	0.768
Glycine	0.86^{c}	0.75^{bc}	0.76^{bc}	0.69^{ab}	0.57^{a}	0.60^{a}	0.028	0.004	<0.001	0.736
Serine	0.92^{c}	0.73^{b}	0.75^{b}	0.70^{b}	0.57^{a}	0.47^{a}	0.037	<0.001	<0.001	0.723
Proline	1.15	1.28	1.17	1.06	0.98	1.00	0.041	0.262	0.044	0.643
Tyrosine	1.48^{c}	1.10^{b}	1.22^{b}	1.08^{b}	1.00^{b}	0.78^{a}	0.056	<0.001	<0.001	0.986
ΣDAA	4.77^{c}	4.35^{bc}	4.25^{bc}	3.91^{b}	3.35^{a}	3.39^{a}	0.137	<0.001	<0.001	0.766
ΣNEAA	8.49^{d}	7.57^{cd}	7.51^{cd}	6.85^{bc}	5.98^{ab}	5.71^{a}	0.257	<0.001	<0.001	0.994

EAA = essential amino acids; DAA = delicious amino acids (including alanine, aspartic acid, glutamic acid and glycine); NEAA = non-essential amino acids.
Values are mean of 3 replicates. $^{a-d}$Values not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

Table 12
The linear variation analysis between free amino acid content of hepatopancreas and the level of CPC substituted fish meal.

Item	Equations	R^2
EAA		
Arginine	$y = -0.7276x + 3.8151$	0.4744
Histidine	$y = -0.3492x + 0.5565$	0.6806
Isoleucine	$y = -0.5251x + 0.8475$	0.7281
Leucine	$y = -1.1365x + 2.1284$	0.7275
Lysine	$y = -1.0794x + 2.6437$	0.6200
Methionine	$y = -0.4343x + 0.8262$	0.6351
Phenylalanine	$y = -0.8394x + 1.4870$	0.6889
Threonine	$y = -0.5105x + 0.7903$	0.8029
Valine	$y = -0.5549x + 1.0903$	0.6870
ΣEAA	$y = -6.1594x + 14.187$	0.7390
NEAA		
Alanine	$y = -0.6762x + 1.6030$	0.8024
Cysteine	$y = -0.113x + 0.1546$	0.8240
Aspartic acid	$y = 0.0038x + 0.2708$	0.0004
Glutamic acid	$y = -0.9219x + 2.0200$	0.7569
Glycine	$y = -0.3638x + 0.8448$	0.6401
Serine	$y = -0.5194x + 0.8848$	0.7829
Proline	$y = -0.3327x + 1.2325$	0.2567
Tyrosine	$y = -0.7473x + 1.3897$	0.6718
ΣDAA	$y = -1.9562x + 4.7352$	0.7860
ΣNEAA	$y = -3.6756x + 8.3989$	0.7925

EAA = essential amino acids; DAA = delicious amino acids (including alanine, aspartic acid, glutamic acid and glycine); NEAA = non-essential amino acids; y = free amino acid content of tissues (mg/g, based on dry matter); x = CPC replacement fish meal levels (%).
Data analysis based on the full values of each treatment.

($P < 0.05$), as shown in Fig. 5B. In crabs fed diets supplemented with CPC, the expression level of carnitine acetyl-transferase (*caat*) was significantly downregulated.

4. Discussion

4.1. Nutritional composition of test ingredients

In the assessment process, ingredient characterization is an often overlooked but it is a crucial step for all nutritional studies (Glencross, 2020). Although the concentrations of some EAA (Ile, Leu, Lys, Met and Thr) of CPC were lower, Arg and Phe concentrations were higher than those of fish meal, and the protein level and TAA concentration were much higher than those of soybean meal and close to those of fish meal. This makes CPC a promising ingredient among the current dietary protein sources. Moreover, the lipid content of CPC was lower than that of fish meal and soybean meal, indicating that more fat needs to be supplemented when using CPC at high levels.

4.2. Apparent digestibility of nutrients in test ingredients by *E. sinensis*

The ADC of dry matter reflects the total level of digestion and absorption of nutrients by animals. A previous study found that the dry matter ADC of plant protein sources was lower than that of most animal protein sources due to their rich cellulose content (Mo et al., 2019), while the dry matter ADC of the tested ingredients in this study ranged from 70.68% to 73.50%, and there was no significant difference among the protein sources. This result indicates that the Chinese mitten crab, as an omnivorous animal, could make good use of the ingredients of legumes and cottonseeds.

Since the quality of dietary protein is an important factor affecting animal growth performance, protein and amino acid ADC is a practical and efficient way for evaluating ingredient. In this study, the protein ADC values of fish meal and soybean meal were 87.21% and 83.16%, respectively, which were generally consistent with those obtained in the study of Chinese mitten crab (Mu et al., 2000). The protein ADC of CPC was above 90%, which was not significantly different from that of fish meal. In a previous study, it was found that the protein ADC of cottonseed meal by the Chinese mitten crab tended to be close to soybean meal (Mu et al., 2000). Compared with the common cottonseed meal, the CPC has the characteristics of high protein and low gossypol through technical processing (Xie et al., 2022), which may be the reason why the protein ADC of CPC in this study was significantly higher than that of soybean meal, or even slightly higher than that of fish meal.

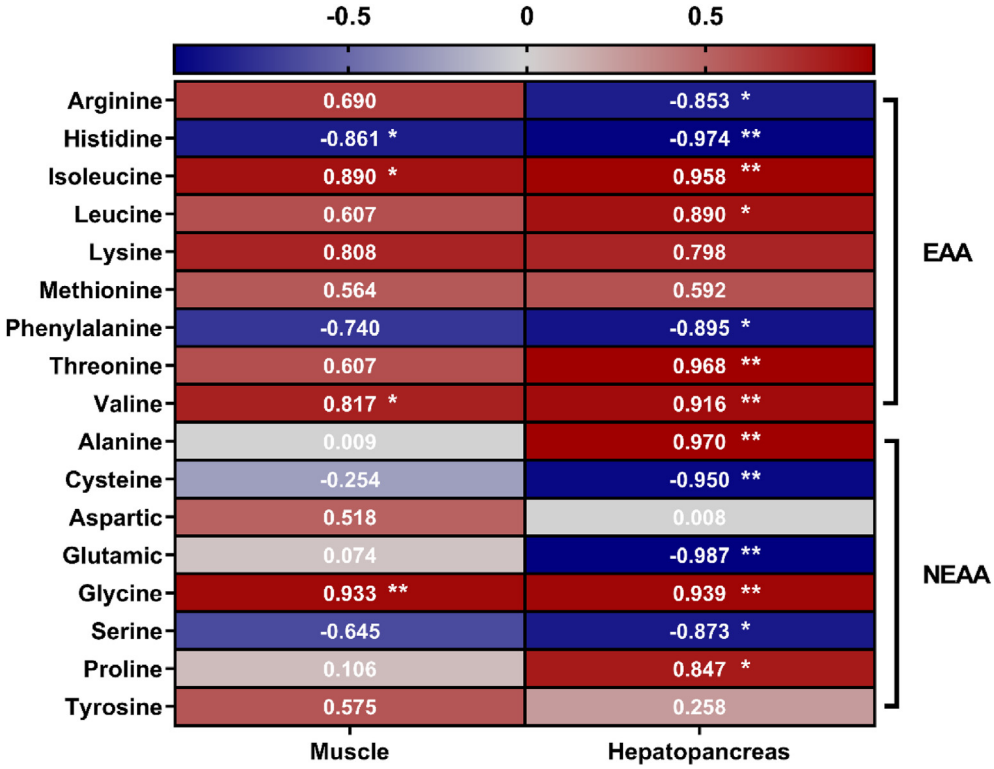

Fig. 3. Pearson correlation coefficient between dietary amino acids and tissue free amino acids of *Eriocheir sinensis*. The independent parameters are dietary amino acids, and the dependent parameters are free amino acids of muscle and hepatopancreas in *Eriocheir sinensis*, respectively. EAA = essential amino acids; NEAA = non-essential amino acids. Values marked with " * " are significantly different by two-tailed test ($P < 0.05$), and values marked with "**" are highly significantly different ($P < 0.01$). It is only possible to conclude that there is a substantial strong correlation between two variables when the correlation coefficient is higher than 0.800 and the *P*-value is less than 0.05.

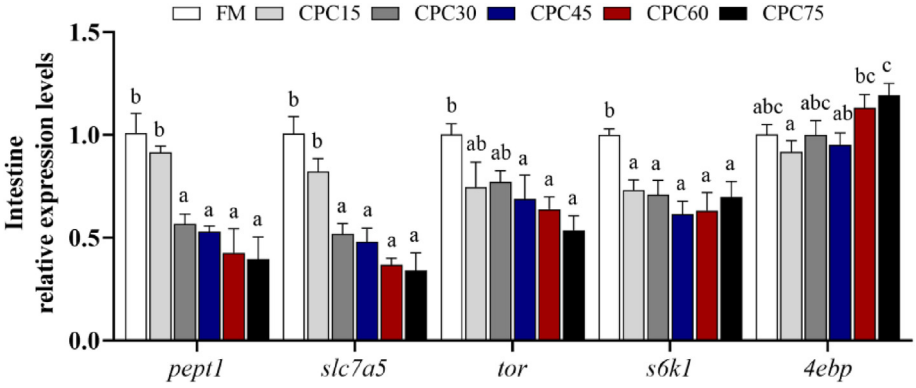

Fig. 4. The expression levels of genes related to nutrient transport and TOR signal pathway in the intestine of *Eriocheir sinensis* fed with different experimental diets for 9 weeks. *pept1* = peptide transporter 1; *slc7a5* = solute carrier family 7 member 5; *tor* = target of rapamycin; *s6k1* = ribosomal protein s6 kinase 1; *4ebp* = eIF4E binding protein; eIF4E = eukaryotic translation initiation factor 4E. $^{a-c}$Bars of the same gene not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

The composition and ADC of amino acids reflect the protein quality of the ingredients. Notably, the ADC values of Ile, Leu, Lys, Met, Thr and Ala of CPC were significantly lower than those of fish meal, suggesting that attention should be paid to the supplementation of these amino acids when using CPC at high levels. However, the ADC of EAA and TAA in CPC were not significantly different from those of fish meal. Therefore, CPC has the potential to replace fish meal as a protein source for crabs from the digestibility point of view, but this needs to be verified in culture experiments.

4.3. Growth performance and feed utilization

Although the protein content of CPC is close to that of fish meal, the Met and Lys contents are much lower than that of fish meal and cannot meet the requirements of crabs. Therefore, all the CPC diets were supplemented with crystal Met and Lys to achieve the same levels as the FM diet. After a 9-week feeding trial, based on the quadratic regression analysis of FCR and PER with dietary CPC content, the optimal replacement levels of fish meal with CPC in crab diets were estimated to be 32.36% and 35.38%, for FCR and PER, respectively. However, when the replacement level of fish meal with CPC was higher than 60%, it had significant adverse effects on growth performance and feed utilization. Additionally, it has been reported that replacement of 60% fish meal with CPC did not significantly affect WG of largemouth bass and hybrid grouper (Cui et al., 2022; Ye et al., 2020). According to previous studies, the addition of feed additives like Lys and Met can mitigate the negative effects of a high plant protein diet (Bulbul et al., 2015; Liu et al.,

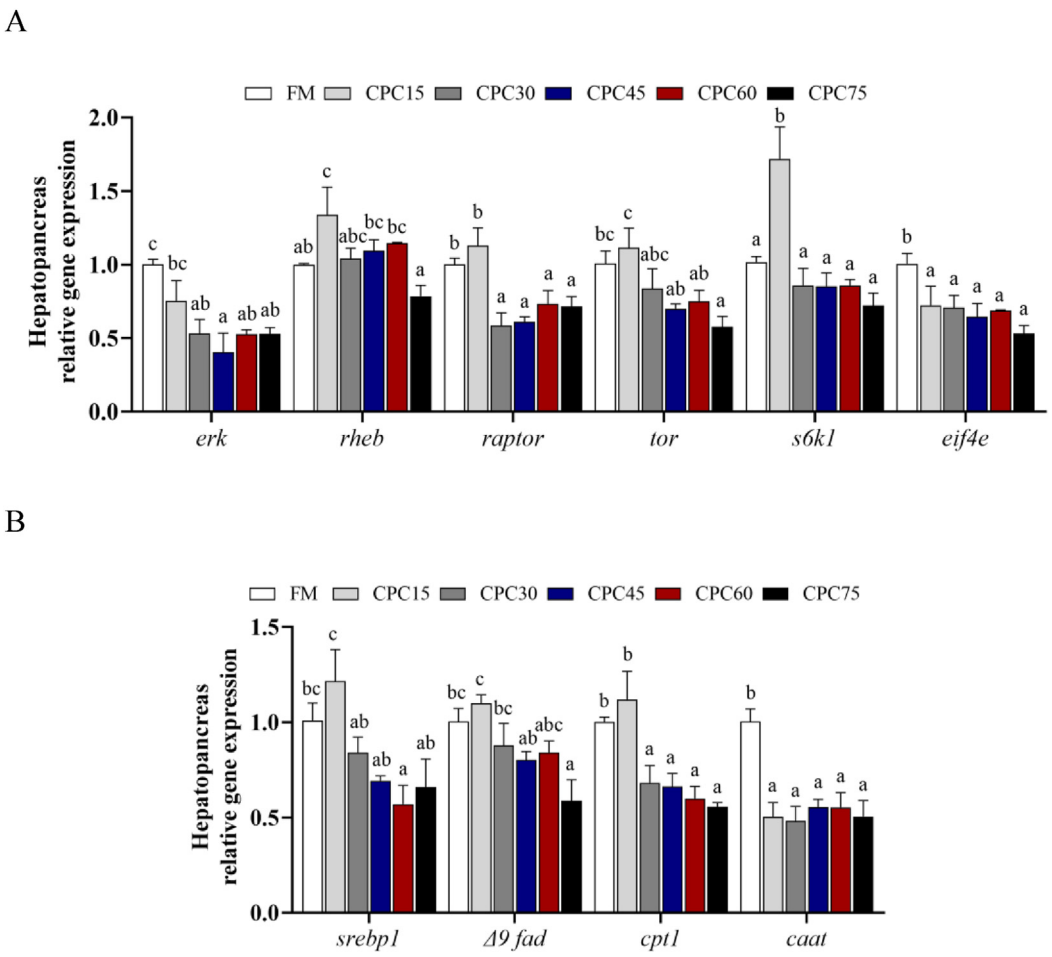

Fig. 5. The expression levels of genes related to TOR signal pathway (A) and lipid metabolism (B) in the hepatopancreas of *Eriocheir sinensis* fed with different experimental diets for 9 weeks. *erk* = extracellular regulated protein kinases; *rheb* = ras homolog enriched in brain; *raptor* = regulatory-associated protein of TOR; *tor* = target of rapamycin; *s6k1* = ribosomal protein s6 kinase 1; *eif4e* = eukaryotic translation initiation factor 4E; *srebp1* = sterol regulatory element-binding protein 1; *Δ9 fad* = Δ9 fatty acyl desaturase; *cpt1* = carnitine palmitoyltransferase 1; *caat* = carnitine acetyl-transferase. $^{a-c}$Bars of the same gene not sharing a common superscript are significantly different by Duncan's test ($P < 0.05$).

2014), and the reason why CPC substitution of 60% fish meal did not affect growth may be due to the supplement of Lys and Met in this study. However, the growth, FCR, PER and PR of the Chinese mitten crab were adversely affected when CPC replaced 75% of fish meal and the inclusion level reached 29.4%. Similar to this, when fish meal was replaced with CPC at 60% (CPC inclusion level 28%) in swimming crabs, both growth performance and feed utilization were negatively impacted (Xie et al., 2022). The results suggest that other factors besides Lys and Met may limit the high addition of CPC. On the one hand, excessive CPC may result in the diet not easy to digest and absorb. Plant is generally rich in fiber, which is not easily digested and absorbed by farm animals and can reduce the retention time of the diet in the intestine, which in turn leads to insufficient absorption of essential nutrients (Gonzalez-Pena et al., 2002; Hansen and Storebakken, 2007; Ren et al., 2020). On the other hand, an excess amount of CPC results in an imbalance of amino acids in the diet. The Arg concentration of CPC was much higher than that of fish meal, and it is possible that the excess Arg antagonized Lys, affecting its metabolism (Wang et al., 2021). In addition to Lys, Met and Arg, there are other amino acids significantly different in concentrations between fish meal and CPC, and the changes of dietary amino acids and tissue free amino acids caused by replacing fish meal with CPC remain to be explored.

4.4. Proximate composition in whole body of crabs

In the present study, there was no significant change in the whole body composition between the crabs fed diets supplemented with CPC and those with FM. Similar results were reported in Pacific white shrimps (Wan et al., 2018) and rainbow trouts (Zhao et al., 2021), which found that the level of dietary CPC replacing fish meal did not affect whole-body composition. Despite the fact that alternate protein sources did not have an impact on the composition of the whole-body (Sudaryono et al., 1995), high CPC levels reduced whole body crude lipid in studies of largemouth bass (Xu et al., 2022) and swimming crabs (Xie et al., 2022). In this study, it may be that the CPC level of 29.40% in the experimental feed did not reach a level that could significantly affect the whole-body composition of crabs, which is consistent with the results of the rainbow trout study (Zhao et al., 2021).

4.5. Biochemical indexes of serum

It is well known that serum and hepatopancreas components such as TP, TG, T-CHO and glucose are correlated with nutrient metabolism and immune response (Zhou et al., 2013). The results of the present study were consistent with those of a study on black

carps, which showed that CPC had no significant effect on serum TP and glucose (Hu et al., 2015). However, our results showed that content of TG in CPC15, CPC30 and CPC45 groups and the content of T-CHO in CPC30 and CPC45 groups were significantly increased. On the whole, the TG and T-CHO contents of juvenile crabs first increased and then decreased with the increase of fish meal replacement by CPC, which is different from the previous study in which that T-CHO content in Pacific white shrimps decreased with increasing CPC levels (Wan et al., 2018). The intake and metabolism of lipids are key factors affecting changes in blood lipids, therefore it is necessary to study this aspect to explore the reasons for the increase in TG and T-CHO concentrations.

The ALT and AST are important enzymes in the hepatopancreas amino acid metabolism. The significant increase in the activities of these two enzymes in serum may indicate a damage of hepatopancreas function (Wang et al., 2014a). In present study, serum ALT activity significantly increased when dietary CPC replaced fish meal at levels above 45%, indicating that hepatopancreas function of the crab was impaired. Previous studies have also reported that excessive CPC in diet could harm cells and tissues (Wan et al., 2018; Xie et al., 2022).

4.6. Antioxidative capacity in hepatopancreas

Reactive oxygen species (ROS), which are frequently formed when substituting fish meal by plant protein sources, can cause oxidative damage that compromises the function of cells and tissues (Liu et al., 2020; Xie et al., 2022; Zhang et al., 2020). Important antioxidant system enzymes include SOD and GPx, of which Cu-Zn SOD is a class of SOD that is mostly present in eukaryotic cells' cytoplasm (Jiang et al., 2016b). When CPC replacement level reached 75% in this study, SOD activity and *gpx* expression were significantly lower than those in the control group. Additionally, as CPC level increased, *cu-zn sod* expression significantly downgraded. The expression of *sod* was significantly up-regulated after CPC replaced 15% fish meal in the study of white shrimp (Wang et al., 2022). In the swimming crab study, CPC substitution of 60% fish meal did not adversely affect expression of *gpx*, and moderate amounts of CPC increased SOD activity (Xie et al., 2022).

Unlike fish, CncC acts as an ortholog of nuclear factor erythroid 2-related factor 2 (Nrf2) in invertebrates, which could regulate antioxidant enzymes and corresponding genes to relieve oxidative stress (Jiang et al., 2016a; Wang et al., 2020). Keap1, the cytosolic repressor of CncC, blocks CncC nuclear transfer and expression (Bayliak et al., 2020; Li et al., 2019). When the amount of T-2 toxin was raised in the study of Chinese mitten crab, the expression of *cncc* first increased and then reduced, whereas the expression of *keap1* exhibited the opposite trend (Wang et al., 2020). In the present study, we found that the expression of *cu-zn sod*, *gpx*, and *cncc* genes was significantly downregulated in the hepatopancreas of crabs in the CPC75 group. These findings suggest that high CPC may reduce the antioxidant capacity of crabs, due in part to its suppression of *cncc* expression levels. Further research is needed to determine the exact mechanism.

4.7. Free amino acid composition of muscle and hepatopancreas

In animals, all amino acids come from feed or body proteins. All NEAA produced by biosynthesis are transported to the free amino acid pool (Yao et al., 2022). The muscle system has the largest pool of free amino acids. Among the EAA, Ile, Leu, Lys, Met and Thr in muscle were significantly affected by the CPC level, all being the lowest in the CPC75 group. This suggests that attention should be paid to the supplementation of Ile, Leu and Thr in addition to the common limiting amino acids (Met and Lys) in CPC applications. Except for His, Ile, Val, and Gly, the variance of free amino acids in muscle did not exhibit a consistent trend with the composition of dietary amino acids, according to a correlation analysis. Similar to the CPC study of the largemouth bass (Xu et al., 2022) and *Clostridium autoethanogenum* protein study of white shrimps (Yao et al., 2022), the composition of muscle free amino acids may be reasonably stable and only marginally influenced by dietary amino acid composition and the digestibility of amino acids. The Ile, Leu, Thr, Val, Ala, Gly, and Pro in hepatopancreas had positive correlations with the relevant amino acids in the diet. Additionally, although the contents of dietary Arg, His, Phe, Cys, Glu, and Ser were on the increase, these amino acids in hepatopancreas decreased, resulting in a significant negative correlation between them. Overall, except Asp and Pro, hepatopancreas free amino acid contents had a negative linear relationship with the dietary CPC level, indicating that hepatopancreas free amino acid contents were more sensitive to replacement level than muscle free amino acid contents.

In addition to growth performance, the meat quality of animals is also a major concern for consumers. Among the free amino acids, DAA are important factors affecting flavor, among which Ala and Gly present sweetness and Glu and Asp present freshness (Chen and Zhang, 2007; Wang et al., 2016). In previous fish meal substitution studies, the reduction of fish meal usually reduced the DAA contents and had an adverse effect on flavor. The concentrations of free Glu, Asp, and Gly in muscle were dramatically reduced in diets for Pacific white shrimps when more than 45% of the fish meal were substituted by *C. autoethanogenum* protein (Yao et al., 2022). Replacing fish meal completely with soy protein concentrate significantly reduced the contents of DAA in grass carp (*Ctenopharygodon idella*) muscle (Yang et al., 2021). In the diets of large yellow croakers (*Larimichthys crocea*), substituting 30% fish meal with Antarctic krill meal significantly reduced the content of Glu, Gly and Ala (Wei et al., 2019). In a CPC study on largemouth bass, Glu and Ala content in muscle increased but DAA contents did not differ significantly (Xu et al., 2022). In the present study, although a moderate amount CPC addition increased Ala content and decreased Asp content, the total DAA content in muscle did not differ considerably from that of the FM group. Unlike fish and shrimp, the hepatopancreas of crabs is also an edible part. In hepatopancreas, when CPC replaced 45% or more fish meal, the contents of Ala, Glu, Gly and total DAA amount were significantly decreased, which may significantly reduce umami and sweet taste. Therefore, CPC substitution of fish meal above 45% can have a negative impact on the flavor of crabs.

4.8. Genes associated with protein transport and TOR signaling pathway in the intestine

The intestine is an important site for digestion, absorption and nutrient exchange of crustaceans, and it is also one of the most closely related parts between organism and the external environment. PEPT1 transported large amounts of amino acids in dipeptides/tripeptides form (Terova et al., 2009; Xu et al., 2020; Zhou et al., 2022). Small peptides are more efficiently absorbed than free amino acids, so PEPT1 has great potential to optimize absorption (Wang et al., 2017). SLC7A5 is a sodium independent high-affinity amino acid transporter, and together with solute carrier family 3 member 2 (SLC3A2) mediates cellular uptake of the large neutral amino acids such as Phe, Tyr, Leu and Trp (Wang et al., 2018). Studies have shown that the TOR signaling pathway can regulate the expression of amino acid and peptide transporters in the intestine of *Caenorhabditis elegans* (Benner et al., 2011) and grass carp (Wang et al., 2018), respectively. The present results show that increasing the dietary CPC level could inhibit intestinal TOR

signaling pathway (down-regulating *tor* and *s6k1* expression and up-regulating *4ebp* expression) and the expressions of *pept1* and *scl7a5*. In the grass carp study, the linear down-regulation of *pept1* and partial amino acid transporter gene expression in various intestinal segments was a significant factor for the decrease of intestinal free amino acids with the increase of dietary gossypol level (Wang et al., 2018). Therefore, in the present study, with the increase of CPC level, the lack of some free amino acids in hepatopancreas and muscle could be attributed to the reduced expression levels of *pept1* and *slc7a5*, resulting in insufficient uptake of amino acids.

4.9. Genes associated with nutrition metabolism in hepatopancreas

In crustaceans, the hepatopancreas not only absorb and store nutrients, but also serve as the metabolic center of the body (Wang et al., 2014b; Zhang et al., 2022). The TOR is a serine/threonine protein kinase member of the phosphoinositide-3-kinase-related kinase family, and is involved in the regulation of several key metabolic mechanisms (Kim and Guan, 2011). TOR signaling pathway, including mTOR complex 1 (mTORC1) and mTORC2, is a major nutrient sensitive pathway that plays an important role in substance metabolism and cell growth. The two complexes differ in function and structure, with the former sensitive to rapamycin and nutrients, while the latter insensitive (Jewell et al., 2013; Magnuson et al., 2012). In this study, the expression levels of *tor*, *s6k1*, *raptor* and *rheb* involved in the TOR signaling pathway were up-regulated first and then down-regulated with the addition of CPC in diets. The results show that appropriate CPC in the diet could activate TOR pathway, which is consistent with the study in swimming crabs (Xie et al., 2022). The dietary content of Arg increased with the increase of CPC level, and studies have shown that an appropriate level of Arg can activate TOR signaling pathway (Chantranupong et al., 2016; Liu et al., 2021; Wang et al., 2021). Therefore, we speculate that the Arg content in the CPC15 diet is beneficial to increase the expression of key genes in the TOR signaling pathway and promote protein synthesis. The reason for the inhibition of TOR signaling pathway with increasing CPC replacement levels may be that the excessive Arg content in diet has an antagonistic effect on Lys, which affects Lys utilization and metabolism. Furthermore, inadequacy of most free amino acids in hepatopancreas may also be an important reason. Therefore, the TOR signaling pathway was downregulated when CPC replaced 75% fish meal, which may have led to reduced growth of crab.

In addition, the nuclear translocation and processing of the SREBPs can be regulated by TOR through S6K1, thereby affecting lipid synthesis (Liu and Sabatini, 2020). SREBP1 and Δ9 FAD were crucial transcription factors for lipogenesis related genes and played a central role in cellular adipogenesis and lipid homeostasis (Arrese and Soulages, 2010; Hishikawa et al., 2020; Wu et al., 2014). In this study, the expression trends of *tor* and *s6k1* were similar to those of *srebp1* and *Δ9 fad*, so the expression changes of lipid metabolism-related genes may be affected by the expression of TOR signaling pathway related genes. Cpt1 and Caat are key enzymes of fatty acid oxidation and catalyze the conversion of fatty acid coenzyme into fatty acid carnitine (Coleman, 2004). In the study of golden pompano, when 60% fish meal was replaced by low-phenolic cotton seed meal (CSM), the expression of lipid anabolism genes was downregulated and the expression of lipid catabolism genes was activated, which may be the reason for the decreased crude lipid and partial fatty acids contents of whole fish (Qin et al., 2021). In the study of Nile tilapia (*Oreochromis niloticus*), high CPC substitution levels downregulated fatty acid synthesis gene expression, up-regulated catabolic gene expression and reduced lipid accumulation in the liver (Wu et al., 2022). However, in this study, high levels of CPC downregulated the expression of lipid anabolism and catabolism genes, which might be attributed to self-regulation to maintain body lipid balance in crabs. These results may provide a reasonable explanation for the absence of changes in crude lipid of whole-crab after replacement of fish meal with CPC.

5. Conclusions

In conclusion, the apparent protein digestibility of CPC is relatively high, which is close to that of fish meal. CPC can be used as a high-quality protein source in the diets of Chinese mitten crabs. Based on the quadratic regression analysis of FCR and PER with the dietary CPC content, the optimal replacement levels of CPC for fish meal were estimated to be 32.36% and 35.38% for FCR and PER, respectively. When the replacement level of fish meal with CPC exceeded 45%, the antioxidant capacity and free amino acids contents in hepatopancreas were significantly reduced. Meanwhile, in the application of CPC, attention should be paid to the supplementation of Ile, Leu and Thr in addition to common limiting amino acids (Met and Lys). Because CPC affects the hepatopancreas free amino acid contents and may affect flavor of crabs, it is not recommended to add CPC to pre-market crab diet.

Author contributions

Xinting Liu conducted the feeding trial, data curation and drafted the manuscript; **Danyang Zou** and **Yizhu Wang** assisted with conducting the feeding trial and sample analysis; **Yutong Zhuang**, **Yang Liu** and **Yanyu Li** helped with sample collection and feed produce; **Zhenzhu Sun** helped with project administration; **Chaoxia Ye** revised the manuscript. All authors read and approved the final manuscript.

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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