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Effect of dietary arginine levels on growth performance, protein synthesis, antioxidant capacity and immunity of postlarval mud crab *Scylla paramamosain*

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This study investigated the effects of different dietary arginine (Arg) levels on the growth, protein synthesis, antioxidant capacity, and immunity of postlarval mud crab *Scylla Paramamosain*. Six isonitrogenous and isolipidic diets were formulated to contain 1.51%, 1.81%, 2.16%, 2.35%, 2.73%, and 3.07% dietary Arg levels (dry matter). There were four replicates for each diet treatment (26 crabs per replicate, initial body weight: 7.40 ± 0.15 mg). After eight weeks of feeding trial, the survival and molting frequency (MF) of crabs were not affected by the experimental treatment ($P>0.05$). Crabs fed the 2.50% Arg diet achieved the highest weight gain (WG) and specific growth rate (SGR) ($P<0.05$). The whole-body protein content of the 2.16% and 2.73% Arg groups were significantly higher than that of the 1.51% Arg group ($P<0.05$). Crabs in the 2.35% group obtained the highest levels of phenylalanine and leucine ($P<0.05$). Superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (T-AOC) activity in the 2.16%, 2.35% and 2.73% Arg groups were significantly higher than that in other treatments ($P<0.05$). Malondialdehyde (MDA) concentration and alkaline phosphatase (AKP) activity were not significantly affected by the treatments. The transcript levels of insulin-like growth factor 1 (*igf-1*), rapamycinin (*TOR*), S6 kinase-polypeptide 1 (*s6k1*) in crabs fed with 2.16% and 2.35% dietary Arg were significantly higher than those in crabs fed with 1.51% and 3.07% dietary Arg ($P<0.05$). The lowest prophenoloxidase (*proPO*), *relish*, and *lysozyme* transcript levels were observed in crabs fed the 1.51% dietary Arg. The current study founded that the Arg requirement for postlaval *S.paramamosain* was 2.34% (5.20% of the dietary protein), based on the second order polynomial regression analysis of WG.

KEYWORDS

arginine, growth performance, protein synthesis, health status, *Scylla paramamosain*

Introduction

Dietary protein and amino acids (AAs) are essential for the optimum health, growth, development, and survival of animals (Herring et al., 2021; Li et al., 2021b). As a basic AA in physiological body fluids, L-Arginine (Arg) is regarded as an essential amino acid (EAA) and functional AA for aquatic animals (Wu, 2014). It takes part in multiple metabolic pathways *in vivo*, including the synthesis of protein, nitric oxide, creatine, proline, glutamate, polyamines, and agmatine (Wu et al., 2009; NRC, 2011). Moreover, Arg and its metabolites are important regulators of key metabolic or cell signaling pathways that are essential for vascular regulation, neurotransmission, nutrient sensing, and anti-inflammatory responses of animals (Reyes et al., 1994; Paudel et al., 2021). Arg is also a potent secretagogue of insulin, growth hormone, and *igf-1*, which can regulate the metabolism of glucose and AAs in various tissues of animals (Newsholme et al., 2005; Wu, 2022). The immune and antioxidant functions of Arg have been extensively reported in different animals (Birmani et al., 2019; Li et al., 2021b).

The functions and requirements of Arg for aquatic animals has been well summarized in recent reviews (Li et al., 2021a; Wu, 2022). Tu et al. (2015) proposed that dietary Arg supplementation could balance AAs and activate the target of *TOR* signaling pathway sensitive to nutrition, thereby promoting protein synthesis in *Carassis auratus gibelio*. Gu et al. (2022) and Liang et al. (2016) showed that the expression of *TOR* and its downstream target *s6k1* in muscle and liver of *Hemibagrus wyckoiides and Megalobrama amblycephala* were improved by dietary Arg supplementation. Like in teleost, Arg is also considered as an essential and functional amino acid in crustaceans (NRC, 2011; Huang et al., 2020). Supplementing an appropriate level of Arg in the diet can enhance growth performance, feed utilization, immune functions, and antioxidant capacity of *Litopenaeus vannamei* (Zhou et al., 2012). Compared to low Arg diet (1.72%), diets contained 2.73-3.07% Arg improved the growth, survival, antioxidant capacity, immunity, and disease resistance of *Eriocheir sinensis* (Qi et al., 2019). Similarly, intraperitoneal injection of Arg stimulated the expression of fch-TOR and activated the *mTOR* signaling pathway in the skeletal muscle of *Fenneropenaeus chinensis* (Sun et al., 2015). On the other hand, excessive Arg intake has a negative impact on animals, resulting in additional energy consumption for deamination and excretion, and ultimately inhibiting growth (Walton, 1985). Therefore, it is necessary to determine the functions of Arg and its dietary optimal level in different crustaceans.

Due to its high nutritional value, short growth cycle, and strong adaptability, the mud crab *Scylla paramamosain* has been considered the most crucial marine aquaculture crustaceans in China (Zheng et al., 2020). Recently, related culture methods and technology have been constantly improved (Zheng et al., 2018). The output of mud crab breeds (mostly *S. paramamosain*) reached 160,616 tons in 2020 (Fishery Bureau of China Agriculture Department, 2020). For mud crabs, dietary requirements of protein, lipid, cholesterol, and phospholipid have also been well studied (Zheng et al., 2018; Xu et al., 2019; Xu et al., 2020; Zheng et al., 2020). To our knowledge, there is limited valuable information on the nutritional and immune aspects of Arg in this species. The lack of relevant information and available data has limited the development of practical formula feeds for *S.paramamosain*. Therefore, it is vital to determine the proper Arg level in diet formulations for postlaval *S. paramamosain*.

Materials and methods

Diet formulation

Six isonitrogenous and isolipidic diets were formulated to contain 1.51%, 1.81%, 2.16%, 2.35%, 2.73%, and 3.07% dietary Arg (L-form, purity ≥98.5%; CJ Biological Technology Ltd., Shenyang, China) levels (dry matter). The basal diet contained 1.51% Arg from fish meal and casein, and the other five diets were prepared by adding L-Arg to increase dietary L-Arg levels with alanine being added to keep the diet isonitrogenous (Table 1). All the dry matters were pulverized by a vertical superfine pulverizer (WFS-8, Zhiyang, Jiangsu), passed through a 125 μm mesh and then thoroughly mixed in a mixer. Crystalline amino acids were prepared by the method provided by Alam et al. (2005). The amino acid mixture was coated with carboxymethyl cellulose (CMC) in water at 60°C. Subsequently, the amino acid mixture was mixed with dry matter. After that, soybean lecithin and oil were added to the mixer and fully mixed. Finally, a suitable amount of water was added to the mixture, which was then made into an even dough. The dough was further processed by a laboratory granulator (G-250, the machine factory of South China University of Technology, Guangzhou, China) into 0.8 mm granules. All prepared feed pellets were dried overnight at 45°C to a constant value and then stored in plastics at -20°C until utilization.

Crabs and experimental conditions

The postlaval crabs were purchased from a commercial hatchery in Taizhou, Zhejiang, China. After a week of acclimatization to laboratory conditions, 624 healthy and similarly sized crabs (7.40 ± 0.15 mg) were chosen and individually placed in cylindrical containers (250 ml). There were four replicates for each diet treatment (26 crabs per replicate). The mud crabs were fed the experimental diet daily at 9:00 and 16:00 respectively. After one hour of the feeding, the

TABLE 1 Formulation and proximate composition of experimental diets (% dry matter).

Ingredients (%)	Dietary Arginine level (%)					
	1.51	1.81	2.16	2.35	2.73	3.07
Fish meal [a]	29.00	29.00	29.00	29.00	29.00	29.00
Casein	10.00	10.00	10.00	10.00	10.00	10.00
Amino acid mixture [b]	13.00	13.00	13.00	13.00	13.00	13.00
Arginine	0.00	0.30	0.60	0.90	1.20	1.50
Alanine	3.07	2.46	1.84	1.23	0.61	0.00
α-starch	24.00	24.00	24.00	24.00	24.00	24.00
Fish oil	3.10	3.10	3.10	3.10	3.10	3.10
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00
Soybean lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin mix [c]	2.50	2.50	2.50	2.50	2.50	2.50
Mineral mix [d]	3.00	3.00	3.00	3.00	3.00	3.00
Cholesterol [e]	0.80	0.80	0.80	0.80	0.80	0.80
Calcium dihydrogen phosphate	1.50	1.50	1.50	1.50	1.50	1.50
Choline chloride	0.70	0.70	0.70	0.70	0.70	0.70
Carboxymethyl cellulose	3.50	3.50	3.50	3.50	3.50	3.50
Cellulose	1.83	2.15	2.46	2.77	3.09	3.40
Proximate composition (dry matter %)						
Moisture	7.42	7.63	6.84	6.79	7.09	7.58
Crude protein	44.96	44.46	44.57	44.41	43.92	44.17
Crude lipid	11.58	10.71	11.62	11.71	11.35	10.71

[a]Purchased from Trident Seafoods Corporation, Seattle, USA.
[b]Amino acid premix, g kg^{-1} mixture: Isoleucine,7.3; Leucine,6.7; Lysine, 12.5; Methionine, 1.5; Phenylalanine, 4.7; Threonine, 7.4; tryptophan, 2.8; Valine, 5.3; Aspartic acid, 18.7; Glutamic acid, 22.8; Serine,3.1; Proline, 11.0; Glycine, 2.5; Alanine, 9.7; Tyrosine, 9.5; cysteine, 4.5.
[c]Vitamin premix, g kg^{-1} mixture: thiamine B1, 5.0; riboflavin, 8.0; nicotinamide, 26.0; biotin, 1.0; calcium pantothenate, 15.0; vitamin B6, 3.0; folic acid, vitamin B9, 5.0; vitamin C, 121.0; vitamin K, 2.02; p-aminobenzoic acid, 3.0; vitamin B12, 1.0; cellulose, 529.0; vitamin A, 25.0; vitamin D3, 25.0; vitamin E, 50.0; inositol, 181.0.
[d]Mineral premix, g kg^{-1} mixture: calcium dihydrogen phosphate, 122.87; lactate, 474.22; sodium dihydrogen phosphate, 42.03; potassium persulfate, 163.83; ferrous sulfate, 10.78; iron citrate, 38.26; magnesium sulfate, 44.19; zinc sulfate, 4.74; manganese sulfate, 0.33; copper sulfate, 0.22; cobalt chloride, 0.43; iodate, 0.02; sodium chloride, 32.33; potassium chloride, 65.75.
[e]Cholestero Purchased from Garden Bio-chem Ltd, Zhejiang, China.

excess feed was removed and the water was changed 100%. Natural indoor lighting was followed and the lighting of each container consistent was kept the same. Death and molts were recorded daily. Water temperature was controlled at 26°C-29°C, the salinity was at 28-29g L^{-1}, the minimal amount of dissolved oxygen was 6 mg L^{-1}, and the concentration of ammonia nitrogen was not more than 0.05mg L^{-1}.

Sampling and analyzing

After eight weeks of trail, all crabs were fasted for 24 hours, and the survival and wet body weight were measured. Ten crabs from each replicate were selected and frozen at -20°C for body composition analysis, and an additional six crabs were selected for dissection to obtain hepatopancreas and muscle for gene expression analysis. In addition, four more crabs were selected for the analysis of antioxidant parameters. All samples were frozen in liquid nitrogen and stored at -80°C.

The proximate composition of diets and crabs' whole-body were analyzed according to standard procedures prescribed in AOAC (1995). Dietary moisture was dried to a constant weight in an oven at 105°C. Whole crab moisture was measured using a freeze dryer (LL1500, Thermo Scientific, Waltham, USA). The Auto Kjeldahl System (K355/K437, Buchi, Flawil, Switzerland) was used to measure the crude protein of feeds and crabs. Total whole-body lipids of crabs were extracted with chloroform/methanol (2/1, v/v) according to the method provided by Folch et al. (1957). To test the amino acid composition of the samples (including experimental diets and whole crab), the amino acid samples were provided to a professional laboratory for measurement using an automatic analyzer (L-8900, HITACHI, Tokyo, Japan; Tables 2, 3).

Whole crab supernatants were prepared for enzymatic activity assays according to the method provided by Xu et al. (2018). Alkaline phosphatase (AKP) activity was analyzed using the methods provided by Wei et al. (2014). Total protein concentration was determined following the method of Bradford (1976). Catalase (CAT) activity was measured using the method provided by Góth (1991). Superoxide dismutase (SOD) activity was measured as described by Zhao et al. (2015). Total antioxidant capacity (T-AOC) was determined using the

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TABLE 2 Amino acids composition of the experimental diets.

Amino acids	Dietary arginine level (%)					
	1.51	1.81	2.16	2.35	2.73	3.07
Threonine	1.11	1.09	1.04	1.16	1.28	1.17
Lysine	2.60	2.50	2.28	2.22	2.11	2.14
Phenylalanine	1.79	1.82	1.76	1.63	1.66	1.75
Arginine	1.51	1.81	2.16	2.35	2.73	3.07
Methionine	1.58	1.61	1.56	1.55	1.50	1.48
Leucine	3.37	3.38	3.22	3.21	3.02	3.14
Isoleucine	2.50	2.47	2.49	2.37	2.50	2.42
Histidine	1.20	1.20	1.14	1.12	1.04	1.14
Valine	1.33	1.27	1.11	1.10	1.12	1.17
Aspartic acid	2.80	2.90	2.97	2.81	2.90	2.93
Glycine	2.07	1.95	1.88	2.08	1.97	1.88
Glutamic acid	7.94	8.04	7.91	7.84	8.08	7.96
Serine	1.80	1.84	1.71	1.65	1.73	1.74
Tyrosine	2.70	2.60	2.62	2.58	2.51	2.59
Proline	1.79	1.72	1.70	1.59	1.70	1.72
Cystine	0.68	0.69	0.65	0.64	0.61	0.63
Alanine	4.97	4.63	4.16	3.63	3.15	2.72

TABLE 3 Amino acid composition (% total amino acids) in whole-body of postlarval *Scylla paramamosain* fed with diets containing different arginine levels.

Amino acids	Diets						SEM[1]	ANOVA[2]	Regression (*P*, R^2)	
	1.51	1.81	2.16	2.35	2.73	3.07		*P* value	Linear	Quadratic
Threonine	4.58	4.58	4.51	4.32	4.22	4.44	0.05	0.142	0.042, 0.175	0.079, 0.215
Lysine	10.06	9.69	9.51	9.55	9.18	9.39	0.10	0.127	0.009, 0.271	0.019, 0.314
Phenylalanine	4.30^{c}	4.21^{bc}	4.00^{ab}	4.25^{bc}	3.87^{a}	3.90^{a}	0.05	0.011	0.003, 0.329	0.015, 0.329
Arginine	6.55^{a}	6.92^{ab}	6.73^{ab}	7.44^{c}	6.99^{b}	6.64^{ab}	0.07	0.002	0.404, 0.032	0.013, 0.340
Methionine	3.27	3.32	3.60	3.93	3.54	3.53	0.07	0.058	0.109, 0.112	0.034, 0.276
Leucine	8.90^{a}	8.99^{ab}	9.69^{c}	9.72^{c}	9.67^{c}	9.59^{bc}	0.11	0.037	0.008, 0.281	0.005, 0.395
Isoleucine	4.82	4.80	4.61	4.71	4.56	4.70	0.05	0.572	0.207, 0.071	0.296, 0.109
Histidine	2.19	2.24	2.14	2.15	2.06	2.26	0.05	0.906	0.870, 0.001	0.785, 0.023
Valine	5.23	5.54	5.68	5.67	5.63	5.62	0.06	0.222	0.064, 0.147	0.032, 0.279
Aspartic acid	11.34	11.08	11.52	10.09	12.23	11.11	0.20	0.058	0.833, 0.002	0.854, 0.015
Glycine	4.66	4.62	4.54	4.61	4.52	4.57	0.05	0.963	0.478, 0.023	0.720, 0.031
Glutamic acid	15.62	15.73	15.54	15.21	15.26	15.94	0.16	0.821	0.968, 0.000	0.598, 0.048
Serine	4.56	4.53	4.38	4.36	4.42	4.46	0.03	0.150	0.105, 0.115	0.028, 0.289
Tyrosine	4.06	4.16	4.47	4.52	4.49	4.50	0.06	0.053	0.004, 0.319	0.005, 0.398
Proline	3.37^{a}	3.41^{ab}	3.57^{bcd}	3.61^{cd}	3.67^{d}	3.47^{abc}	0.03	0.010	0.032, 0.192	0.003, 0.432
Cystine	0.20	0.19	0.21	0.22	0.21	0.21	0.01	0.846	0.469, 0.024	0.723, 0.030
Alanine	6.27^{c}	6.00^{c}	5.30^{a}	5.66^{b}	5.50^{ab}	5.67^{b}	0.08	0.000	0.004, 0.318	0.000, 0.610

Means in the same row with different superscripts are significantly different (P< 0.05).
[1] SEM: pooled standard error of means.
[2] ANOVA: one-way analysis of variance.

method provided by Benzie and Strain (1996). Malondialdehyde (MDA) concentrations were determined following the method of Zhao et al. (2015). All parameters were determined using a commercial kit (Nanjing Jianchen Bioengineering Institute, Nanjing, China) and a microplate reader (Multiskan Go, Thermo Scientific, Waltham, USA).

Gene expression

Total RNA was extracted from hepatopancreas and muscle using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified on a 2.0% agarose electrophoresis to assessing RNA concentration. cDNA transcribed from RNA was synthesized using PrimeScript™ RT Kit (Perfect Real Time; Takara, Dalian, China) according to the protocol provided by the manufacturer. The transcription level of Insulin-like growth factor 1 (*igf-1*), Rapamycinin (*TOR*), S6 kinase-polypeptide 1 (*S6K1*), prophenoloxidase (*proPO*), *relish* and *lysozyme* genes were determined by a Real-Time PCR System (QuantStudio™ 6 Flex, Life Technologies, Carlsbad, USA) according to Zheng et al. (2018). Quantitative real-time PCR was performed using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Since β-actin is relatively stable, it was used as a reference gene (Table 4). The transcription level of different genes was calculated using the $2^{-\Delta\Delta CT}$ equation according to Livak and Schmittgen (2001). Each treatment was performed in quadruplicate and replicated three times on each sample.

Statistical analysis

After all data were tested for homogeneity and normal distribution with Levene's test and Kolmogorov-Smirnov test, respectively, one-way analysis of variance (ANOVA) was used to determine treatment effects, and treatment deviations were ranked using Duncan's multiple range test. Linear and quadratic regression models were constructed to test the correlation of results with dietary Arg levels. The statistical significance level was determined at 5% ($P<0.05$). All parameters were analyzed by SPSS 24.0 (IBM, Chicago, USA) software, and the data were expressed as mean values (n = 4). The second-order polynomial regression model (Robbins et al., 1979) was used to estimate the appropriate supplementation of dietary Arg for *S. paramamosain* on the basis of WG.

Results

Survival and growth performance

After eight weeks of feeding trial, the survival of postlarval *S. paramamosainin* ranged from 76.24% to 96.52% (Table 5). Different dietary Arg levels significantly affected the growth performance of *S. paramamosain*. The highest WG and SGR values were observed in the 2.35% dietary Arg level group ($P<0.05$). Quadratic regression analysis of WG% against dietary Arg levels indicated that optimal dietary Arg level was 2.34% of dry matter (5.20% of dietary protein) for *S. paramamosain* (the optimum is the maximum requirement)

TABLE 4 The sequences of primers in this study.

	Sequence(5'to3')	Production size	Reference	Amplification efficiency
igf-1-F	TCATCACCATCGGCAATGA	237	According to the sequence design of Chinese Mitten Crab (*Eriocheir sinensis*)	104.2%
igf-1-R	TTGTAAGTGGTCTCGTGGATG			
TOR-F	TGACCTCGCCCTAGTGCTT	90	KY608020.1	96%
TOR-R	ATCCAACGGTCACATGCCACA			
s6k1-F	CGCCCCTCAGATTTCCAGT	175	According to the sequence design of swimming crab (*Portunus trituberculatus*)	101.4%
s6k1-R	TCTCAGCCTTTGTGTGCG			
proPO-F	CACTGGCATCTTGTCTACCCT	111	KP710954.1	103.2%
proPO-R	CAGGCGATCCATGTCATAACGA			
relish-F	CAGGTACACCTTTGTGACCGT	100	Zhu et al. (2019)	101.4%
relish-R	CCTTCTACTTAGGGCATTTCG			
lysozyme-F	TGCCATCAACCACCACAACT	196	Xie et al. (2019)	100.9%
lysozyme-R	CCCCTTTCCCTTCCACTTCT			
β-actin-F	GAGCGAGAAATCGTTCGTGAC	183	Zheng et al. (2020)	102.3%
β-actin-R	GGAAGGAAGGCTGGAAGAGAG			

TABLE 5 Growth, feed utilization and morphometrical parameters of postlarval *Scylla paramamosain* fed with diets containing different arginine levels.

	Diets						SEM[1]	ANOVA[2]	Regression (*P*, R^2)	
	1.51	1.81	2.16	2.35	2.73	3.07		*P* value	Linear	Quadratic
Survival[3]	83.10	85.03	92.52	82.83	85.58	76.24	1.95	0.310	0.299, 0.049	0.128, 0.178
FBW[4]	0.16^{c}	0.21^{b}	0.25^{a}	0.28^{a}	0.27^{a}	0.18^{c}	0.01	0.000	0.110, 0.112	0.000, 0.696
WG[5]	2020.81^{c}	2796.00^{b}	3261.74^{a}	3723.73^{a}	3500.49^{a}	2289.09^{c}	139.85	0.000	0.156, 0.089	0.000, 0.778
SGR[6]	5.08^{c}	5.61^{b}	5.86^{ab}	6.07^{a}	5.97^{a}	5.30^{b}	0.08	0.000	0.158, 0.088	0.000, 0.788
MF[7]	4.34	4.49	4.44	4.57	4.65	4.58	0.06	0.806	0.166, 0.085	0.362, 0.092

Means in the same row with different superscripts are significantly different (P < 0.05).
[1] SEM: pooled standard error of means;
[2] ANOVA: one-way analysis of variance.
[3] Survival (%) = 100 × (final amount of crabs)/(initial amount of crabs);
[4] Finial body weight (FBW, g crab-1);
[5] Weight gain (WG, %) = 100 × ((FBW-IBW)/IBW);
[6] Specific growth rate (SGR, % day-1) = 100× (ln (FBW) – ln (IBW))/days;
[7] MF: molting frequency = (Σ moulting times of every survival crab)/final amount of crabs.

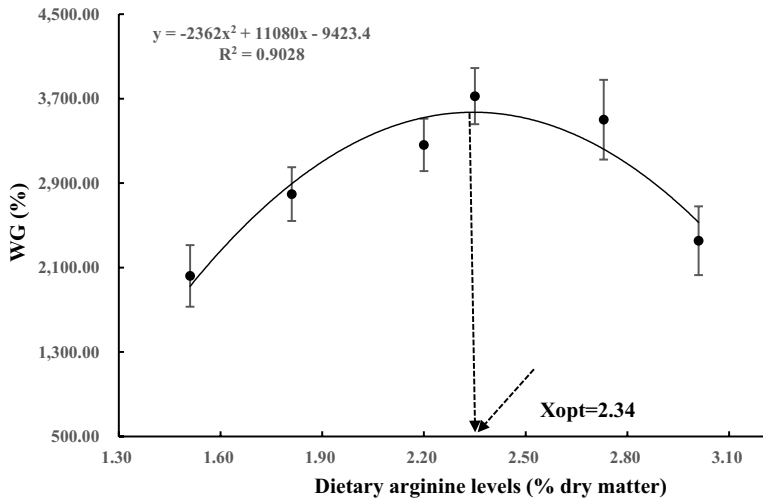

FIGURE 1
Relationship between the weight gain (WG, %) with the dietary arginine levels, where Xopt represents the optimal dietary Arg level for the maximum WG.

(Figure 1). Dietary Arg levels had no significant effect on molting frequency (MF; $P>0.05$).

Whole-body proximate composition

The effects of different treatments on the crude lipid content of crabs were not significant (Table 6). The whole-body protein content of the 2.16% and 2.73% Arg groups were significantly higher than that of the 1.51% Arg group ($P<0.05$). In addition, the effects of different treatments on the content of most AAs in the whole body were not significant, but phenylalanine, Arg, leucine and proline showed linear and quadratic responses to dietary Arg levels ($P<0.05$).

Antioxidant and immunity parameters

In this experiment, dietary Arg levels significantly affected the antioxidant capacity of crabs (Table 7). The highest SOD activity were observed in the 2.35% Arg group ($P<0.05$). The lowest CAT and T-AOC activity were observed in the 1.51% Arg group ($P<0.05$). Although MDA and AKP concentrations were not affected by the experimental treatments, the highest MDA

TABLE 6 Whole-Body composition in postlarval *Scylla paramamosain* fed with diets containing different arginine levels (in % of wet weight basis).

	Diets						SEM[1]	ANOVA[2]	Regression (*P*, R^2)	
	1.51	1.81	2.16	2.35	2.73	3.07		*P* value	Linear	Quadratic
Whole body										
Moisture	74.56	74.20	77.04	77.31	77.46	76.11	0.47	0.160	0.062, 0.149	0.052, 0.246
Protein	8.59^{b}	8.97^{ab}	9.48^{a}	9.10^{ab}	9.39^{a}	8.93^{ab}	0.09	0.035	0.170, 0.084	0.011, 0.352
Lipid	2.13	2.11	2.13	2.14	2.14	2.10	0.02	0.975	0.838, 0.002	0.812, 0.020

Means in the same row with different superscripts are significantly different (P< 0.05).
1 SEM: pooled standard error of means.
2 ANOVA: one-way analysis of variance.

TABLE 7 Antioxidant parameters in the whole-body of postlarval *S. paramamosain* fed the experiment diets for 8 weeks.

	Diets						SEM[1]	ANOVA[2]	Regression (*P*, R^2)	
	1.51	1.81	2.16	2.35	2.73	3.07		*P* value	Linear	Quadratic
SOD[3]	65.52^{c}	67.93^{c}	72.01^{bc}	85.02^{a}	80.86^{ab}	63.03^{c}	2.18	0.004	0.391, 0.034	0.009, 0.360
CAT[4]	1.47^{c}	1.84^{b}	2.72^{a}	2.68^{a}	2.53^{a}	1.84^{b}	0.10	0.000	0.071, 0.141	0.000, 0.846
T-AOC[5]	1.93^{b}	2.12^{a}	2.16^{a}	2.14^{a}	2.14^{a}	1.98^{b}	0.02	0.000	0.453, 0.026	0.000, 0.814
MDA[6]	3.72	3.59	2.83	2.84	2.68	3.09	0.27	0.855	0.292, 0.050	0.405, 0.082

Means in the same row with different superscripts are significantly different (P < 0.05).
[1] SEM: pooled standard error of means.
[2] ANOVA: one-way analysis of variance.
[3] Superoxide dismutase.
[4] Catalase.
[5] Total antioxidant capacity.
[6] Malondialdehyde.

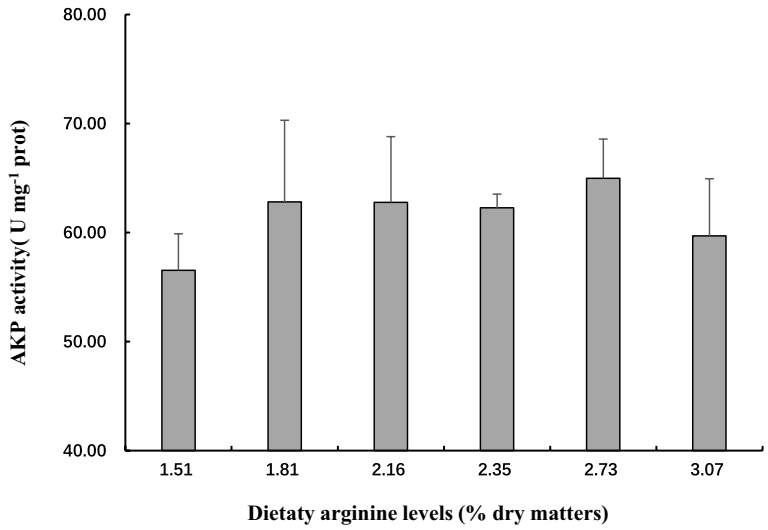

FIGURE 2
Activity of AKP in the hepatopancreas of postlarval *S. paramamosain* fed diets with different arginine levels.

concentration and the lowest AKP concentration were observed in the 1.51% Arg group (P>0.05; Figure 2).

The mRNA expression of *igf-1*, *proPO*, *relish* and *lysozyme* in hepatopancreas as well as *s6k1*, *TOR* in muscle

In this experiment, dietary Arg levels significantly affected transcript levels of *igf-1*, *TOR*, *s6k1*, *proPO*, *relish*, and *lysozyme* ($P < 0.05$; Figures 3–8). The transcript levels of *igf-1*, *TOR*, *s6k1* in crabs fed with 2.16% and 2.35% dietary Arg were significantly higher than those in crabs fed with 1.51% and 3.07% dietary Arg ($P < 0.05$). In addition, the lowest expression of *proPO*, *relish*, and *lysozyme* were observed in the 1.51% dietary Arg group ($P < 0.05$).

Discussion

A high survival rate is necessary for the success of crustacean farming. Cannibalism is the main reason for the low survival rate of crustacean species in nutritional research experiments (Castine et al., 2008). Generally, it is acceptable that the survival rate exceeds 80% in the feeding trial for crustaceans (Catacutan, 2002). In this study, the survival of postlarval mud crab *Scylla paramamosain* ranged from 76.24% to 92.52% among different treatments. A similar survival (from 76.19% to 92.86%) was also recorded in a previous study of *S. paramamosain* (Zheng et al., 2020). This result indicated that the experimental conditions were suitable for the culture of postlarval *S. paramamosain* in this study.

The growth performance of *S. paramamosain* was significantly affected by different feed treatments. With the dietary Arg level increased to 2.35% (5.22% of the dietary protein), the WG value of *S. paramamosain* showed a general increased trend, and then decreased significantly as the dietary Arg level was further increased. A similar tendency was also observed in other species, such as Pacific white shrimp (*Litopenaeus vannamei*; 4.77% of the dietary protein; Zhou et al., 2012). The deficiency of dietary Arg is characterized by poor growth, low feed utilization, or high mortality (Wilson, 2003). Similarly, the SGR and WG values of the 1.51% and 1.81% Arg groups were significantly lower than those of the 2.35% and 2.73% groups. This is in agreement with the results of juvenile *Penaeus monodon* (Millamena et al., 1998). On the other hand, crabs fed with the 3.07% Arg diet had significantly lower WG and SGR values than crabs fed with the 2.35% and 2.73% Arg diets. And the survival rate of crabs fed with the 3.07% Arg diet was also the lowest among all groups. In agreement, Fournier et al. (2003) found that excess dietary Arg would adversely impact the growth performance of *Oncorhynchus mykiss* and *Psetta maxima*. Based on the second order polynomial regression analysis of WG, the optimum dietary Arg level is 2.34% of the dry diet, which is equivalent to 5.20% of the dietary protein. These values are similar to those reported Arg requirements of other crustaceans, such as *Penaeus monodon* (5.30% of dietary protein; Millamena et al., 1998) and *Marsupenaeus japonicus* (5.32% of dietary protein; Alam et al., 2004). However, it is estimated that the *Eriocheir sinensis* has a higher requirement for Arg (9.18% of dietary protein; Ye et al., 2010). A possible explanation is that water-soluble crystalline AAs were used in the experiment, and the long feeding time of Chinese mitten crabs easily led to the loss of some AAs, so that the real content of AAs in the feed were lower than the values assumed in the experimental design. In contrast, some other crustaceans have lower requirements for Arg (4.2-4.7% of dietary protein; *Palaemonetes varians*; Palma et al., 2015). This may be attributable to the fact that the experimental diets were pelletized

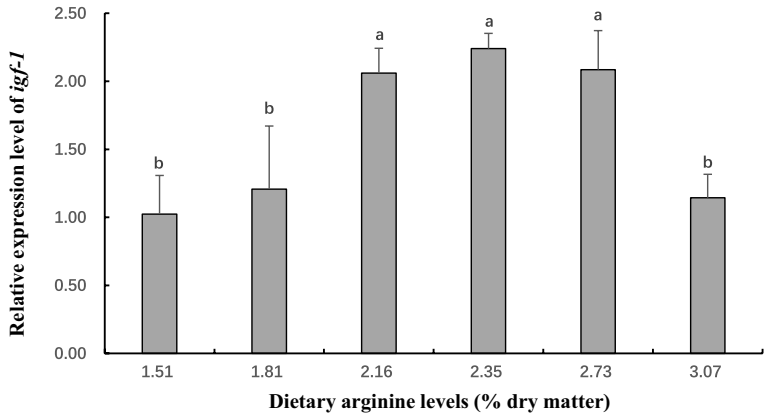

FIGURE 3
The gene expression (Insulin-like growth factor 1 (*igf-1*) in hepatopancreas) of postlarval *Scylla* paramamosain fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

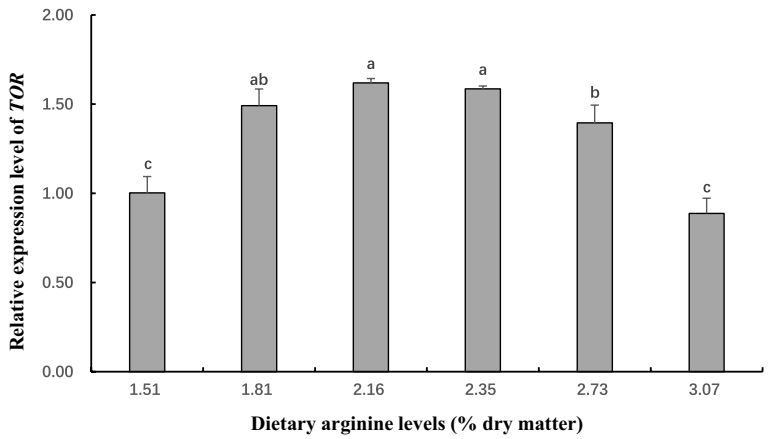

FIGURE 4
The gene expression (Rapamycinin (*TOR*) in muscle) of postlarval *Scylla paramamosain* fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

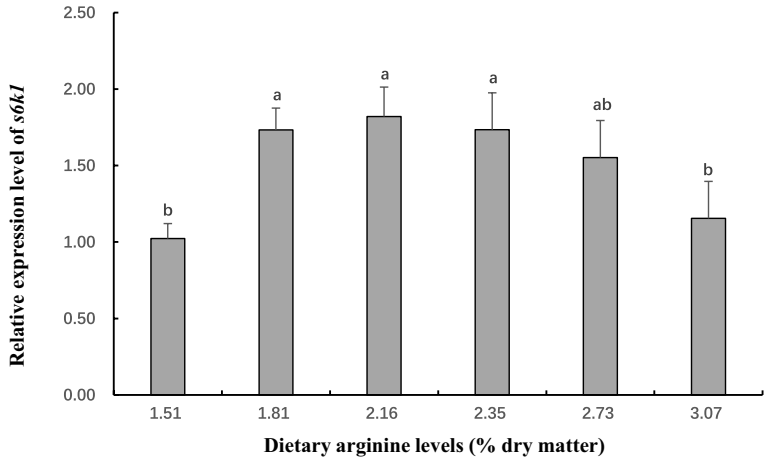

FIGURE 5
The gene expression (S6 kinase-polypeptide 1 (*s6kl*) in muscle) of postlarval *Scylla paramamosain* fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

with steam, which resulted in apparently highly water-stable pellets and a low level of dry matter losses. Therefore, the Arg requirements for crustaceans is dependent on different species, size, dietary protein sources and levels, feeding practices, rearing conditions, and experimental design (NRC, 2011).

An imbalanced proportion of EAAs can reduce the absorption and utilization of AAs by animals. In the present study, the content of Arg, leucine, phenylalanine and proline in carb were significantly increased with increasing the dietary Arg level from 1.51% to 2.35%. Similar results were also found in *Megalobrama amblycephala and Oncorhynchus mykiss* (Yamamoto et al., 2000; Ren et al., 2013). However, no further increases were observed in crab fed with diets containing Arg beyond 2.35%. A possible explanation is that the deamination of Arg was up-regulated in these groups, and excess Arg was excreted through soluble ammonia, as previously reported on *Paralichthys olivaceus* (Alam et al., 2002). Excess Arg intake also causes additional energy consumption for deamination and excretion (Walton, 1985).

Insulin-like growth factor 1 (*igf-1*), also called somatomedin C, is a hormone with a molecular structure similar to insulin. *igf-1* plays an important role in growth and anabolism (Philipps et al., 1988). *igf-1* level is a very useful indicator for measuring the fish growth and response to change in feed nutrient

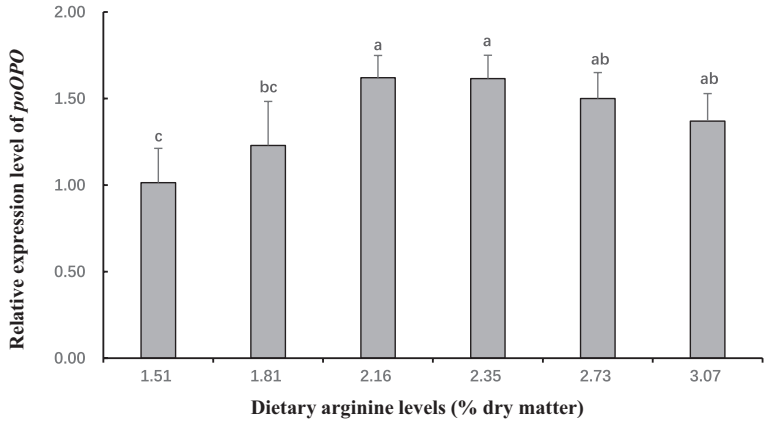

FIGURE 6
The gene expression (prophenoloxidase (*proPO*) in hepatopancreas) of postlarval *Scylla paramamosain* fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

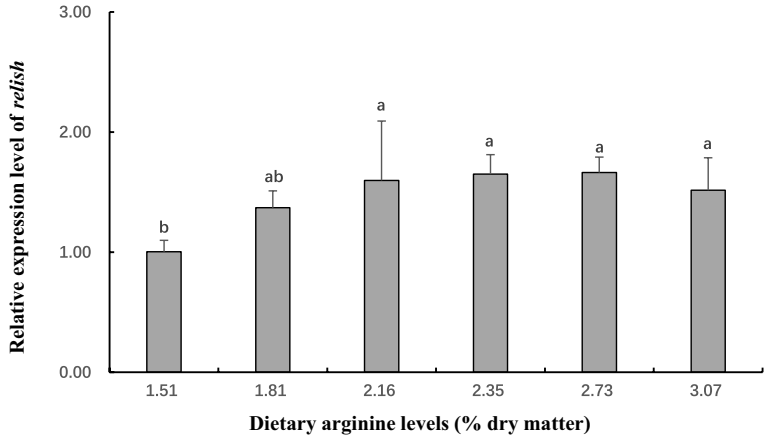

FIGURE 7
The gene expression (*relish* on hepatopancreas) of postlarval *Scylla paramamosain* fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

composition (Picha et al., 2008; Izutsu et al., 2022). Dietary Arg promotes hepatic *igf-1* transcription, and its positive effect growth has been reported in some fish species, such as *Ictalurus punctatus* (Pohlenz et al., 2013), *Sparus aurata* (De Celis et al., 2004) and hybrid *Epinephelus fuscoguttatus*♀×*Epinephelus lanceolatus*♂ (Wu et al., 2018). In this study, the mRNA expression of *igf-1* in hepatopancreas showed a significantly increase with increasing dietary Arg content to 2.35%. However, excess dietary Arg (3.07%) decreased the mRNA expression of *igf-1*. It has reported that excessive dietary Arg leads to insulin resistance through a negative feedback mechanism, resulting in phosphorylation of serine/threonine *in vivo*, thereby affecting the transcription level of *igf-1* (Harrington et al., 2004; Um et al., 2006). These results suggest that appropriate dietary Arg levels could activate *igf-1* to enhance the growth of *S. paramamosain*.

Mu et al. (2020) and Gu et al. (2022) reported that Arg could decrease the inhibition of translation and increase TOR activity, resulting in improve the synthesis of proteins in *Cromileptes altivelis* and *Hemibagrus wyckoiides*. Furthermore, TOR signaling pathway is also activated by a combination of insulin and AAs (Serrana and Johnston, 2013), and not just by AAs in *Oncorhynchus mykiss* (Lansard et al., 2010). In addition, Hara et al. (1998) found that in the absence of growth factors such as insulin, the presence of substantial amounts of AA can greatly promote the phosphorylation of *s6k1*, a key substrate of *mTOR*.

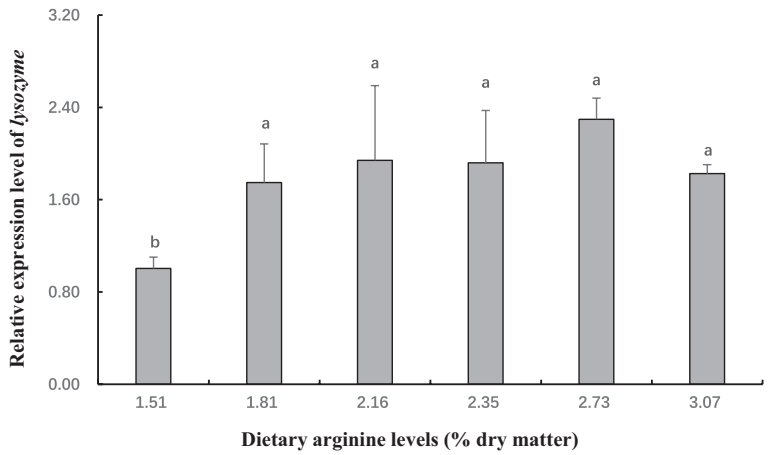

FIGURE 8
The gene expression (*lysozyme* in hepatopancreas) of postlarval *Scylla paramamosain* fed diet with different arginine levels. Means in the same row with different superscripts are significantly different (P< 0.05).

Similarly, Fingar et al. (2002) claimed that *TOR* increases the translation of 5' TOP mRNA through phosphorylation of *s6k1* to control cell size. Interestingly, the study revealed that overexpression of *s6k1* alone enhanced cell size in the absence of rapamycin, whereas they worked together to further increase cell size if co-expressed. In this experiment, dietary Arg levels significantly affected the transcription level of *TOR* and *s6k1* mRNA, which showed a similar trend with whole body protein content in *S. paramamosain*. Similar results were also reported in the studies on *Portunus trituberculatus* (Jin et al., 2016). These results indicated that appropriated dietary Arg could activate *TOR* signaling pathway, which could boost protein synthesis in this species. Similarly, optimal dietary Arg levels could also improve the transcription level of *TOR* and *s6k1* in *Cromileptes altivelis* (Mu et al., 2020) and *Ctenopharyngodon idellus* (Chen et al., 2019). However, when the level of dietary Arg exceeded the optimum level (i.e., 3.07%), the transcription level of *TOR* and *s6k1* were be significantly reduced. This phenomenon can be explained by the fact that Arg overload negatively affects insulin signaling through *mTOR*/*s6k1* phosphorylation, leading to insulin resistance (Um et al., 2006; Wullschleger et al., 2006). The specific mechanism of Arg activation of the *TOR* signaling pathway in crustaceans is complex and needs to be further studied.

Phosphatases and antioxidant enzyme systems are important components of the non-specific immune system and have been widely used to assess the health of crustaceans (Wei et al., 2014; Dong et al., 2018; Fu et al., 2022). Phosphatase participated in many metabolic processes, including chitin secretion, calcium absorption, and calcium phosphate deposition (Robertson, 1937; Travis, 1955; Kobayashi et al., 1983). In this study, AKP activity was highest at 2.73% dietary Arg levels. Furthermore, antioxidant enzymes (e.g., SOD, CAT, and T-AOC) play a direct role in removing excess ROS and protecting cells from oxidative damage (Ke et al., 2011; Ighodaro and Akinloye, 2018). T-AOC can be used as a comprehensive index to assess the antioxidant capacity of the body. In this experiment, crab fed appropriated dietary Arg level (1.81-2.73%) have higher T-AOC activity, indicating the crab had better antioxidant ability. Similarly, the highest SOD activity was observed in crab fed diets with 2.5% Arg. Malondialdehyde (MDA) produced by endogenous oxidative damage *in vivo* is one of the final metabolites of lipid peroxidation, which can reflect the extent of lipid peroxidation and the extent of cell injure (Grotto et al., 2009). The lowest MDA concentration was observed in the treatment group with appropriate dietary Arg levels. These findings suggested that an adequate Arg content in diet can improve the health of *S. paramamosain*. Juvenile mud crabs must adapt to various environments from offshores to estuaries and settle on tidal flats or mangrove fringes, experiencing large variations in salinity throughout the entire process. Previous research found that salinity changes stimulate the production of ROS (Wang et al., 2022). Therefore, we suggest that an appropriate dietary Arg level (2.35% group) has benefits for early juvenile *S. paramamosain*.

Crabs lack adaptive immunity and must rely on innate immunity, to defend themselves against infectious pathogens (Iwanaga and Lee, 2005; Tran et al., 2020). As a crucial component of innate immunity, the prophenoloxidase (*proPO*) activating system is unique to invertebrates (Cerenius et al., 2008; Chen and Wang, 2019; Tran et al., 2020). In this study, the highest expression of *proPO* was observed at dietary Arg levels of 2.16% and 2.35%. This indicates that an adequate amount of Arg can boost the immunity of *S. paramamosain* by stimulating the *proPO* system. Similarly, it was reported that appropriate Arg levels (2.73% and 3.72%) in the diet can up-regulate *proPO* mRNA

expression to increase the immunity and disease resistance of *Eriochier sinensis*. On the other hand, the activation and translocation of NF-kB are linked to the expression antibacterial peptide in invertebrates. Peroxinectin is an MPO homologue that can promote the nuclear translocation of the nuclear factor NF-kB (Lau et al., 2005; Lin et al., 2007). Therefore, we examined the gene expression of relish, an NF-B-like transcription factor, as well as antibacterial peptide genes such as lysozyme. The results showed that an appropriate dietary Arg level could increase the expression levels of relish and lysozyme. In summary, supplementing the appropriate Arg content in the diet can boost the immunity of *S. paramamosain*.

Conclusion

The current study founded that the dietary Arg requirement for postlaval *S.paramamosain* was 2.34% (5.20% of the dietary protein). Meanwhile, appropriate dietary Arg levels significantly increased the transcription level of *igf-1*, *TOR*, and *s6k1*-related genes, whereas excessive dietary Arg levels suppress the transcription of these genes. Furthermore, this study suggested that appropriated dietary Arg levels improves the antioxidant capacity as well as the immune system of postlarval *S.paramamosain*. Finally, future research is suggested to explore the specific mechanism behind the regulatory effect of arginine on the immunity in *S.paramamosain*, which will help with the development of a balanced diet with EAAs and the development of feed formulation for commercial purposes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by Institutional Animal Care and Use Committee of Zhejiang Ocean University.

Author contributions

DW designed the experiments with the help of JW. DW performed the experiments and drafted the manuscript. TH provided the experimental laboratory to ensure that the experiments could be carried out properly. WF and TH supervised the experimental procedure. HX and XYL analyzed the data. DW, XL and JW revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author XL is employed by Guangdong Yuehai Feeds Group Company Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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