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Arginine supplementation improves growth, antioxidant capacity, immunity and disease resistance of juvenile Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

To investigate the effects of arginine (Arg) on the growth, antioxidant capacity, immunity and disease resistance of juvenile Chinese mitten crab, three diets containing Arg levels at 1.72% (control), 2.73% and 3.72% were formulated and fed to Chinese mitten crab (0.22 ± 0.03 g) for eight weeks. The weight gain, ecdysterone and growth hormone in the serum, relative expression of insulin-like growth factor 2 in the hepatopancreas significantly increased in crabs fed the 2.73% and 3.72% Arg diets. The protein and lipid contents significantly increased in crabs fed the 3.72% Arg diet. The feed conversion ratios in crabs fed the diets with Arg additions were lower than in the control. Arg supplementation also enhanced the antioxidative capacity by increasing the activities of superoxide dismutase, catalase and the relative expression of Kelch-like ECH-associated protein 1 gene in the hepatopancreas, which subsequently decreased malondialdehyde content in the hepatopancreas. Besides, Arg also decreased nitric oxide content in the serum and the activity of nitric oxide synthetase in the hepatopancreas. The relative mRNA levels of crustin, relish, lysozyme and cryptocyanin genes were significantly upregulated by Arg supplementation. The activities of acid phosphatase and alkaline phosphatase in the serum significantly increased in crabs fed the 3.72% Arg diet than those in the control. Similarly, the relative mRNA levels of crustin, cryptocyanin and proPO genes were significantly upregulated in crabs fed the 2.73% Arg diet after lipopolysaccharide challenge, and in crabs fed the 3.72% Arg diet after the Poly (I:C) challenge. The crabs fed the 2.73% and 3.72% Arg diets had higher survival rate after bacterial infection than those fed the control diet. This study indicates that the addition of Arg to the diet at 2.7–3.7% can improve the growth, survival, antioxidant capacity, immunity and disease resistance in juvenile Chinese mitten crab.

1. Introduction

Arginine (Arg) is an essential amino acid of aquatic animals and has a wide range of biological functions [1–3]. It is not only one of the important amino acids for protein synthesis, but also a precursor for the synthesis of polyamine, creatine and nitric oxide [4–7]. Arg serves as a potent stimulant of insulin [8] and growth hormone [9] and plays an important role in animal metabolism and growth regulation [3,10]. Dietary supplemented with 1%–2% Arg significantly improved the growth of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) [11]. Similarly, the optimal dietary Arg level (2.73%–2.74%) achieved maximal growth of juvenile golden pompano (*Trachinootus ovatus*) [12]. Like in teleost, Arg is also an important component in crustaceans. When dietary Arg was not sufficient in the Pacific white shrimp (*Litopenaeus vannamei*), growth performance was compromised [13]. The similar results have been also reported in megalopae [14] and juvenile Chinese mitten crab [15]. However, most studies have mainly focused on the requirements for growth performance of crustaceans, but the study on immunity in crustacean is rare.

Arg is closely related to the immune function [4]. For instance, Arg can resist intestinal *Escherichia coli* infection of mice through immune regulation [6]. Because Arg can be decomposed to produce nitric oxide

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(NO) in nitric oxide synthase (NOS) to regulate immune function [16]. In mammals, NO can mediate toll-like receptor signaling pathways and pass biological signals to the nuclear factor as the kappa-light-chain-enhancer in B cells (NF-kB) to release cytokines for immune regulation [17]. In fish, Arg can improve immunity via regulating the inflammatory response [18] and the TOR pathway [19]. In addition, Arg also can improve specific and non-specific immunity in fish by regulating plasma cytokines such as C3 and IgM [16,19]. However, crustaceans are relatively inferior to mammalian and fish species in evolution, and many physiological functions are different between vertebrates and invertebrates. Invertebrates solely rely on innate immune system including cellular and humoral immunity to defend invading microbes [20,21]. Where the prophenoloxidase (proPO) system is one of the important components of innate immunity in invertebrates [22]. Moreover, peroxinectin is a cell adhesion factor related to proPO [23], and can interact with the proPO system and perform the immunoregulation [24]. Besides, peroxinectin is a homologue of human myeloperoxidase (MPO), and influences nuclear translocation to regulate the expression of antibacterial peptide in immune signaling pathways [24]. Unfortunately, little research has been done on the effect of Arg on the immune system of crustaceans. In other invertebrate species such as mosquito (*Anopheles stephensi*), Arg can regulate the immune function through the nitric oxide pathway [25]. However, it is unclear how Arg regulates the immunity function in crabs.

Chinese mitten crab (*Eriocheir sinensis*) is one of the most important crustaceans in freshwater aquaculture because of its high economical value and preference by consumers due to its high nutritional value and unique flavor [26]. Therefore, its production in China has dramatically increased in recent years, reaching over 750,000 tons in 2017 [27]. Regrettably, the culture of Chinese mitten crab has faced many challenges such as pathogen infections and unexpected environmental stress like pH, dissolved oxygen and ammonia [28,29]. These stresses may cause crab mortality and result in economic loss to farmers. Hence, the development of methods to reduce farming risks, such as cultivation of robust and disease resistant crabs through nutrition manipulation are highly required. Arg can improve the immunity and disease resistance of aquatic animals [16,18]. However, the effects of Arg on immunity and disease resistance in Chinese mitten crab are currently unknown. Therefore, this study explored the role of Arg in immunity and disease resistance of Chinese mitten crab. To achieve this objective, we fed Chinese mitten crab with three doses of Arg for eight weeks and determined the growth, antioxidant capacity and immune response. Furthermore, at the end of the feeding trial, the crabs were challenged with *Aeromonas hydrophila* to assess the ability of disease resistance in crabs.

2. Material and methods

2.1. Experimental diets

The formulation and chemical proximate composition of experimental diets are shown in Table 1. Three isonitrogenous and isolipidic diets (control diet, 2.73% Arg diet and 3.72% Arg diet) were formulated to contain 1.71%, 2.73% and 3.72% Arg by gradient adding crystalline L-arginine at the expense of crystalline glycine. The actually measured quantity of amino acids of the experimental diets is shown in Table 2.

All ingredients were ground into power and sieved through a 60-mesh strainer, weighed according to the formulation, and then mixed all ingredients thoroughly with an electric mixer, subsequently oil and distilled water were added to make a dough and then pelleted with a screw-press pelletizer (F-26, South China University of Technology, Guangzhou, China) using 2.0 mm die. Pellets were air dried at room temperature for approximately 48 h to the moisture content < 10%. After drying, all diets were packed in bags and stored at −20 °C until the diet was fed to carbs.

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

Ingredients	1.72% Arg (Control)	2.73% Arg	3.72% Arg
Fish meal[a]	15.00	15.00	15.00
corn gluten meal	15.00	15.00	15.00
Casein[b]	12.00	12.00	12.00
Gelatin[c]	4.00	4.00	4.00
Corn starch[d]	28.00	28.00	28.00
Soybean lecithin[e]	1.00	1.00	1.00
Cholesterol	0.30	0.30	0.30
Fish oil	2.00	2.00	2.00
Soybean oil[f]	2.00	2.00	2.00
Glycine[e]	2.10	1.10	0.10
Cellulose	6.20	6.20	6.20
Methionine[e]	0.30	0.30	0.30
Lysine[e]	0.50	0.50	0.50
Sodium carboxymethyl cellulose	2.00	2.00	2.00
Choline chloride[e]	0.50	0.50	0.50
Vitamin premix[g]	3.00	3.00	3.00
Mineral premix[h]	3.00	3.00	3.00
Betaine[e]	3.00	3.00	3.00
Butylated hydroxytoluene	0.10	0.10	0.10
Arginine[e]	0.00	1.00	2.00
Proximate analysis			
Moisture	10.23	10.12	10.25
Crude protein	39.26	39.95	39.29
Crude lipid	6.95	6.73	6.67
Ash	6.21	6.19	6.41

[a] Fish meal: TASA steam dried fishmeal, Tecnologica De Alimentos S. A, Peru.
[b] Casein: Gansu hualing dairy Co Ltd, Gansu, China.
[c] Gelatin: Baotou Dongbao Bio-Tech Co Ltd, Baotou, China.
[d] Corn starch: Beijing gusong food Co Ltd, Beijing, China.
[e] Sangon Biotech (Shanghai) Co Ltd, Shanghai, China.
[f] Soybean oil: Cofco food marketing Co. Ltd, Beijing, China.
[g] Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; pyridoxine hydrochloride, 0.225 g; *para*-aminobenzoic acid, 0.1 g; ascorbic acid, 0.5 g; biotin, 0.005 g; folic acid, 0.025 g; cholecalciferol, 0.0075 g; α-tocopherol acetate, 0.5 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g [30].
[h] Mineral premix (per 100 g premix): KH_2PO_4, 21.5 g; NaH_2PO_4, 10.0 g; $Ca(H_2PO_4)_2$, 26.5 g; $CaCO_3$, 10.5 g; KCl, 2.8 g; $MgSO_4{\cdot}7H_2O$, 10.0 g; $AlCl_3{\cdot}6H_2O$, 0.024 g; $ZnSO_4{\cdot}7H_2O$, 0.476 g; $MnSO_4{\cdot}6H_2O$, 0.143 g; KI, 0.023 g; $CuCl_2{\cdot}2H_2O$, 0.015 g; $CoCl_2{\cdot}6H_2O$, 0.14 g; calcium lactate, 16.50 g; Fe-citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g [30].

Table 2
Amino acid composition of experimental diets (Dry matter, %).

Amino acid	1.72% Arg (Control)	2.73% Arg	3.72% Arg
Arginine	1.72	2.73	3.72
Alanine	2.34	2.30	2.30
Asparagine	2.69	2.65	2.64
Glutamic acid	6.75	6.58	6.61
Glycine	3.91	3.07	2.26
Histidine	1.04	1.03	1.10
Isoleucine	1.58	1.55	1.54
Leucine	3.62	3.52	3.55
Lysine	2.65	2.62	2.59
Methionine	1.04	0.95	1.20
Phenylalanine	1.77	1.72	1.74
Serine	1.71	1.68	1.67
Threonine	1.39	1.37	1.37
Tyrosine	1.51	1.46	1.49
Valine	1.98	1.93	1.93

2.2. Feeding trial, sampling and growth evaluation

All animal care and handing procedures in this study were conducted under the Guidance of the Care and Use of Laboratory Animals

in China and were approved by the Animal Care Committee of East China Normal University.

The experiment was carried out at the Experimental Base of Zhejiang Freshwater Fisheries Research Institute (Huzhou, China). Juvenile crabs were obtained from Shanghai Ocean University (Shanghai, China). Prior to the trial, all of crabs were stocked in 300 L tanks (100 × 80 × 60 cm), and fed with commercial diets to acclimatize for one week. Crabs were fasted for 24 h before the start of the feeding trial and then 720 crabs (0.22 ± 0.01 g, mean ± S.E.) with intact appendages were randomly distributed into 18 tanks with 40 crabs each in six replicates. Four bundles of corrugated plastic pipes, each bundle consisted of five pipes (12 cm long and 25 mm diameter), and four arched tiles were placed in each tank as shelters to reduce attacking behavior. Diets with a daily ration of 4% body weight were hand-fed to crabs three times daily, 07:00 (20%), 16:00 (20%) and 24:00 (60%). Feces were removed in the morning (09:00 h) after feeding, and the water of 30% tank volume was exchanged daily. The experimental water was filtered by a quartz sand filter (Xinyi Water Treatment Equipment Factory, Huzhou, China) and aerated fully before adding into the trial tanks. Any dead crabs were immediately removed from the tank, weighed and recorded. Feed intake of each tank was recorded accurately throughout the trial period. During the experimental period, the daily water temperature varied from 25 °C to 28 °C. Dissolved oxygen was above 7 mg L^{-1} and ammonia below 0.05 mg L^{-1}.

At harvest, all the crabs from each tank were anesthetized with crushed pieces of ice, and then counted and group-weighed by tank after fasting for 24 h. Five crabs from each tank were euthanized and sealed in bags at −20 °C for whole-body proximate analysis. Another 10 crabs per tank were euthanized and then the hemolymph was drawn out immediately from the leg joints of each crab with a 1.0 mL syringe without anticoagulants. The hemolymph was placed in a 4 °C refrigerator for 5 h, and then the coagulated hemolymph was stirred up with a syringe needle and centrifuged in a high-speed refrigerated centrifuge for 10 min (8000 r/min). Serum was carefully collected with a pipette and kept at −80 °C until analysis. Concurrently, hepatopancreas samples were put into liquid nitrogen immediately and then stored at −80 °C for the analyses of enzyme activities and gene expressions.

Weight gain, feed conversion ratio and survival were calculated according to the following formulas:

Weight gain (WG, %) = $(W_t - W_0) \times 100 / W_0$; Feed conversion ratio (FCR) = feed intake / $(W_t - W_0 + W_d)$; Survival (%) = 100 × final number of crab / initial number of crab;

Where W_t, W_0 and W_d were final crab weight, initial crab weight and dead crab weight, respectively.

2.3. Disease resistance test

For lipopolysaccharide (LPS) injection, three crabs from each tank (18 crabs per group) were randomly selected and injected with 10 μg/g LPS (Sigma-Aldrich, L2630) [31]. After 12 h post-injection, six crabs were randomly collected from each group (n = 6) and their hepatopancreas were sampled for RNA extraction. The post-injection sampling time was chosen according to the previous study reported by Jin et al. [31] and our preliminary experiment. The same number of crabs were also injected with Poly (I:C) at a dose of 5 μg/g Poly (I:C) (APExBIO, B5551) [32] as described before and sampled at 12 h post-injection according to the study by Du et al. [32].

After sampling, the challenge test was conducted according to the description by Sun et al. [33]. Gram negative bacterium *Aeromonas hydrophila* was obtained from East China Normal University, Shanghai, China. After final harvesting, 40 crabs were randomly selected from each treatment and then injected with 1.0×10^7 *A. hydrophila*. Survival rates were recorded at 0, 6, 12, 24, 36, 48 and 96 h, respectively.

2.4. Sample analysis

2.4.1. Chemical composition analysis

Chemical compositions of the experimental diets and crab whole body were determined by standard procedures using proximate composition analysis [34]. Samples were dried at 105 ± 5 °C and then stored in a glass desiccator to cool down to room temperature (25 °C). The samples were repeatedly weighed on an electronic balance (BSA223S, Sartorius Scientific Instruments (Beijing) Co., Ltd., China) until the sample weight reached a constant value. Subsequently, samples were pulverized and stored in a glass desiccator at room temperature (25 °C) for the analyses of crude protein, crude lipid and ash content. Crude protein was determined by the Kjeldahl method (N × 6.25) using Kjeltec™ 8200 (Foss, Hoganas, Sweden). Crude lipid was quantified by Soxhlet extraction method using a 1000 mL Soxhlet extraction tube (Fujian minbo toughened glass Co. Ltd., Fujian, China). For ash content analysis, samples were burned in a carbonization furnace (Zhejiang lichen instrument technology Co., Ltd) at 300 °C and then placed in a muffle furnace (PCD-E3000 Serials, Peaks, Japan) at 550 °C for 6 h. For total amino acid content analysis, diets were freeze-dried overnight, and then hydrolyzed for 24 h in 6 N HCl at 110 °C. After pretreatment, all the samples were analyzed with an L-8900 amino acid analyzer (Hitachi, Tokyo, Japan).

2.4.2. Analysis of antioxidant capacity and immune enzyme activities

The method of hepatopancreas sample treatment was described by Han et al. [30]. The antioxidant capacity and immune factors were measured using the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the instructions of the manufacturer. Among which, the activities of acid phosphatase (ACP; Cat. No. A060-2), alkaline phosphatase (AKP; Cat. No. A059-2) and the content of nitric oxide (NO; Cat. No. A012-1) were determined in the serum. The activities of superoxide dismutase (SOD; Cat. No. A001-1), nitric oxide synthase (NOS; Cat. No. A014-1-1), catalase (CAT; Cat. No. A007-1-1), the content of malondialdehyde (MDA; Cat. No. A003-1), total antioxidant capacity (T-AOC; Cat. No. A015-2) and total protein (TP; Cat. No. A045-2) were determined in the hepatopancreas.

2.4.3. Analysis of ecdysterone and growth hormones in serum

The contents of ecdysterone and growth hormone in the serum were measured using ELISA kits (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) on the crab in accordance with the instructions of the manufacturer.

2.4.4. Analysis of gene expression by qRT-PCR

Total RNA was extracted from the hepatopancreas of Chinese mitten crab using Trizol (RN0101, Aidlab, China) according to the manufacturer's protocol. The total RNA concentration and quality were estimated using a Nano Drop 2000 spectrophotometer (Thermo, USA). If the ratio of A260/A280 was between 1.8 and 2.0, the sample was used for reverse transcription using a PrimeScript™ RT master mix reagent kit (Perfect Real Time, Takara, Japan). The specific primers for the genes of *E. sinensis* were designed based on the transcriptome sequencing results and NCBI data base using NCBI Primer BLAST (Table 3). The RT-PCR amplification reactions were performed in a volume of 10 μL, containing 5 μL 2 × SYBR Premix Ex TaqTM, 0.25 μL of 10 mM forward primer, 0.25 μL of 10 mM reverse primer and 4.5 μL of diluted cDNA, using CFX96 Real-Time PCR system (Bio-rad, Richmond, CA). PCR conditions were as follows: 94 °C for 3 min, and following 40 cycles at 94 °C for 15 s and 60 °C for 50 s, and 72 °C for 20 s. Samples were run in triplicate and normalized with the control gene β-actin. The gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ comparative C_T method [35].

Table 3
Primer sequences used for real-time PCR.

Primers name	Sequences (5′-3′)	Production size	Reference
Kelch-like ECH-associated protein 1 F	GGCGGTGGTGAACAGACTTA	106 bp	c138113_g1_i1
Kelch-like ECH-associated protein 1 R	CACAAGGGTCCACTGGTTGT		
Relish F	ACAACAGCCGAAGGAGTCAG	112 bp	GQ871279.1
Relish R	AGACACTCAGCAGCACTCAC		
Cryptocyanin F	CAACGACGACATCAAGCTGC	101 bp	JX162648.1
Cryptocyanin R	CATAACCGTGAGCAATGGCG		
Prophenoloxidase F	CCATGTCATCATTGCAGCGG	119 bp	EF493829.1
Prophenoloxidase R	TGTACTTGTGCCAGCGGTAG		
Crustin F	ACCACCCAAAACATGCTCCA	90 bp	FJ974138.1
Crustin R	GGCTTGCAGACATGTTCACC		
Lysozyme F	ATGATGCGTGTGATCTGCCT	111 bp	JN416111.1
Lysozyme R	CATGACGCATCCATCGCTTG		
Insulin-like growth factor 2 F	AAGTCCTGGAAAGCGTTGG	82 bp	Liu et al. [36]
Insulin-like growth factor 2 R	GTCACATGCACCACCTGTGT		
Peroxinectin F	CAGCAACGACTACAACCCGA	91 bp	GU353176.1
Peroxinectin R	TCCTTGCACCAGGGAATGAC		
β-actin F	TGGGTATGGAATCCGTTGGC	101 bp	KM244725.1
β-actin R	AGACAGAACGTTGTTGGCGA		

2.5. Statistical analysis

Statistical analysis was performed using the SPSS 23.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA). The data were represented as the mean ± standard error (S.E.). All data were subject to normality test and homogeneity of variance by using Shapiro-Wilk and Levene's equal variance tests, respectively. If the data show a normal distribution, one-way analysis of variance (ANOVA) was used, and when the means of each treatment were significant differences, Duncan's multiple range test was used to compare means among all the treatments. Significance was set at $P < 0.05$.

3. Results

3.1. Growth, feed efficiency and body composition

Crabs fed the 2.73% Arg diet or 3.72% Arg diet showed higher weight gain and lower feed conversion ratio than those fed the control diet ($P < 0.05$, Fig. 1). Significantly higher survival rate was found in crabs fed the 3.72% Arg diet than those in the control ($P < 0.05$). Crabs fed the 3.72% Arg diet obtained the highest crude protein content, which was significantly higher than those fed the control diet ($P < 0.05$). Crude lipid showed a similar trend to the crude protein, and the whole-body lipid content of crabs fed the 3.72% Arg diet was significantly higher than the others ($P < 0.05$). The contents of ecdysterone and growth hormone in the serum were significantly higher in crabs fed the 2.73% Arg and 3.72% Arg diets than those fed the control diet ($P < 0.05$). The relative expression of IGF2 was significantly upregulated in crabs fed the 2.73% Arg diet and 3.72% Arg diet ($P < 0.05$).

3.2. Antioxidative capacity and oxidative stress

The MDA content in the hepatopancreas of crabs fed the control diet was significantly higher than those fed the other two diets ($P < 0.05$) and MDA content in the hepatopancreas of crabs fed the 2.73% Arg diet was significantly higher than those fed the 3.72% Arg diet ($P < 0.05$, Fig. 2). The activity of T-SOD in the hepatopancreas of crabs fed the 2.73% Arg diet was significantly higher than those fed the control and the 3.72% Arg diets ($P < 0.05$), and there were no significant differences between the control diet and the 3.72% Arg diet ($P > 0.05$). Activities of catalase in the hepatopancreas of crabs fed the 2.73% Arg diet and 3.72% Arg diet were slightly higher than in the control crabs ($P > 0.05$). No significant differences were obtained in T-AOC among all the treatments ($P > 0.05$). The content of nitric oxide in the hemolymph of crabs fed the control diet was significantly higher than those fed the other two diets ($P < 0.05$). The activities of total nitric oxide synthetase (TNOS) and inducible nitric oxide synthase (iNOS) in the hepatopancreas of crabs fed the control diet were significantly higher than those fed the other two diets ($P < 0.05$). The relative mRNA expression of KEAP 1 in the hepatopancreas was significantly upregulated in crabs fed the 3.72% Arg diet ($P < 0.05$).

3.3. Immunity

No significant differences were found in the relative mRNA expression of proPO among all the treatments ($P > 0.05$, Fig. 3). The significantly higher expression of peroxinectin was found in crabs fed the 2.73% Arg diet than those fed the other diets ($P < 0.05$). The relative mRNA expressions of crustin and relish in the hepatopancreas of crabs fed the 2.73% Arg and 3.72% Arg diets were significantly upregulated compared with the control crabs ($P < 0.05$). The relative mRNA expressions of lysozyme in the hepatopancreas of crabs fed the 2.73% Arg and 3.72% Arg diets were significantly upregulated compared with the control crabs ($P < 0.05$). The relative expressions of cryptocyanin in the hepatopancreas of crabs fed the 2.73% Arg and 3.72% Arg diets were significantly higher than the control crabs ($P < 0.05$). The activity of acid phosphatase in the serum of crabs fed the 3.72% Arg diet was significantly higher than those fed the control and the 2.73% Arg diets ($P < 0.05$), and there were no significant differences on acid phosphatase activity between the control diet and the 2.73% Arg diet ($P > 0.05$). The activities of alkaline phosphatase in the serum of crabs fed the 2.73% Arg and 3.72% Arg diets were significantly higher than those fed the control diet ($P < 0.05$).

3.4. Disease resistance

Crabs fed the 2.73% Arg diet had significantly higher mRNA levels of proPO, crustin and cryptocyanin in the hepatopancreas than in the control crabs with post LPS injection of 12 h ($P < 0.05$, Fig. 4). In addition, crabs fed the 3.72% Arg diet only obtained significantly higher proPO expression than those in the control crabs. However, for Poly (I:C) injection, the relative expressions of proPO, crustin and cryptocyanin in the hepatopancreas of crabs fed the 3.72% Arg were significantly higher than those in the control crabs ($P < 0.05$). Death began at 12 h after *Aeromonas hydrophila* challenge. After 96 h, the survival rates of crabs fed the 2.73% Arg or 3.72% Arg were 32.5% and 27.5%, respectively. However, the survival of crabs fed the control diet was only 5% at 96 h.

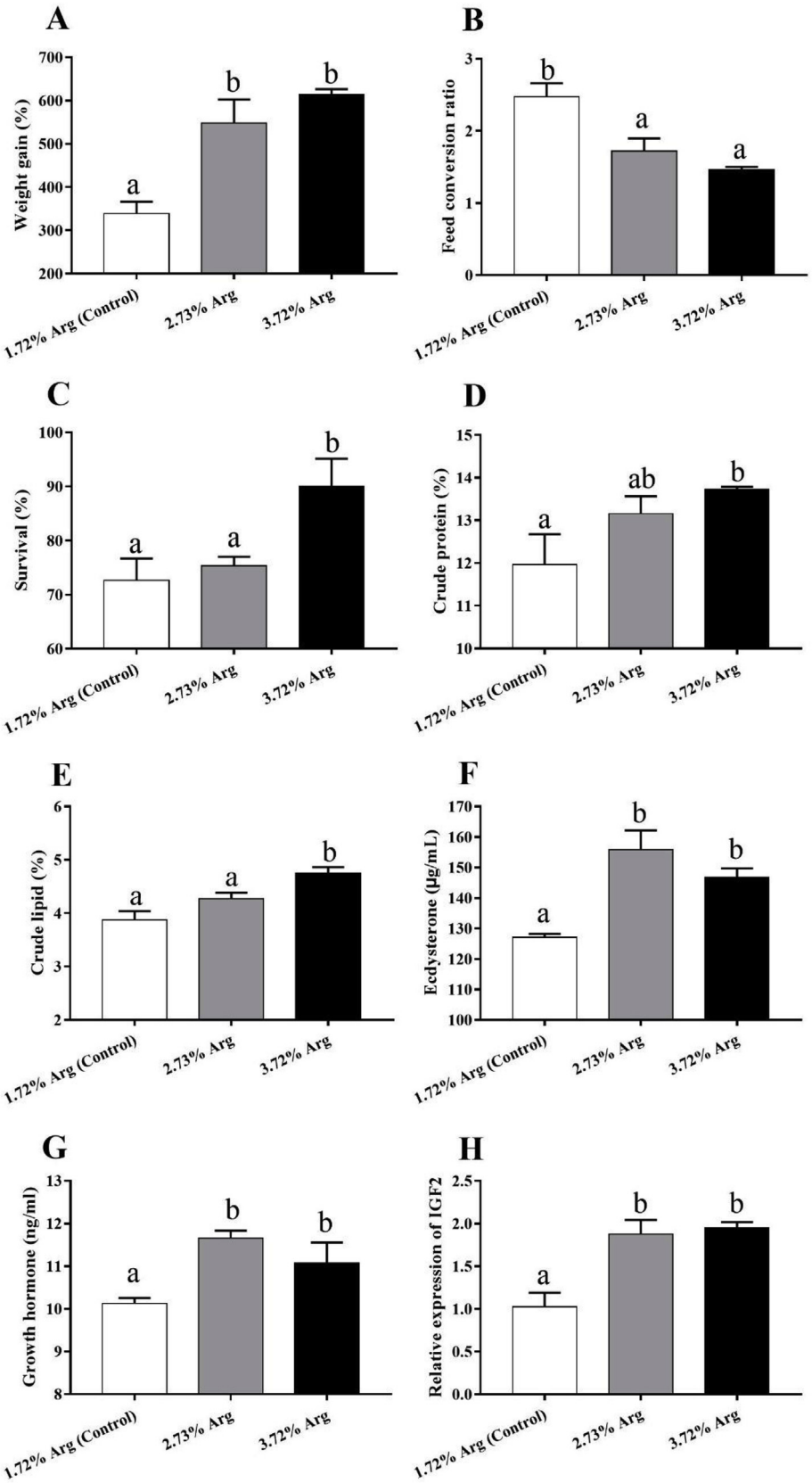

Fig. 1. Effects of arginine on the growth performance, feed efficiency and body composition of Chinese mitten crab. (A) Weight gain, (B) Feed conversion ratio, (C) Survival, (D) Crude protein of the whole body, (E) Crude lipid of the whole body, (F) Ecdysterone in the hemolymph, (G) Growth hormone in the hemolymph, (H) Relative expression of IGF2 in the hepatopancreas. Different lowercase letters show significant differences ($P < 0.05$).

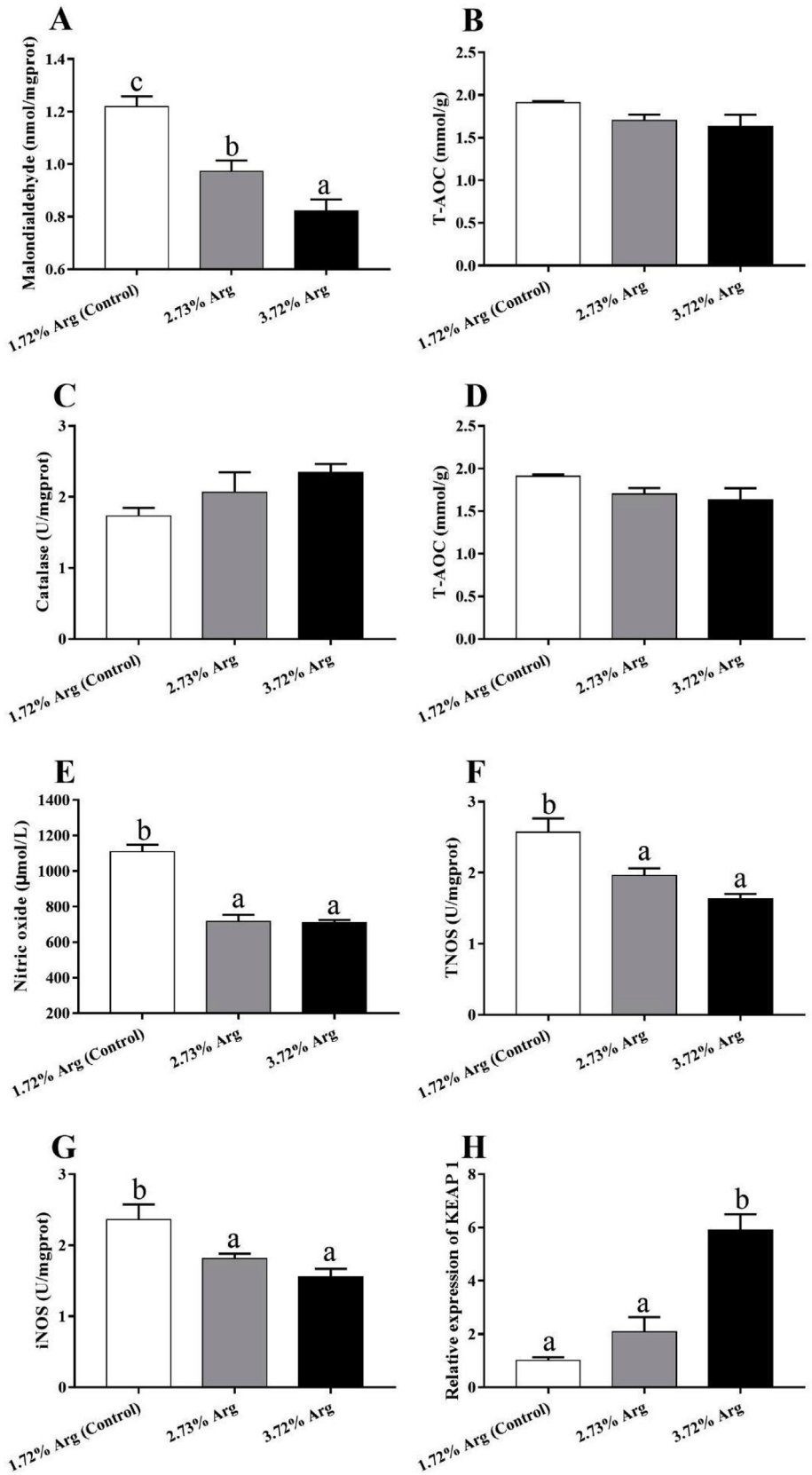

Fig. 2. Effects of arginine on the antioxidative capacity and oxidative stress of Chinese mitten crab. (A) Malondialdehyde in the hepatopancreas, (B) T-SOD: Total superoxide dismutase in the hepatopancreas, (C) Catalase in the hepatopancreas, (D) T-AOC: Total antioxidant capacity in the hepatopancreas, (E) Nitric oxide in the hemolymph, (F) TNOS: Total nitric oxide synthetase in the hepatopancreas, (G) iNOS: Inducible nitric oxide synthase in the hepatopancreas, (H) Relative expression of KEAP 1 in the hepatopancreas. Different lowercase letters show significant differences ($P < 0.05$).

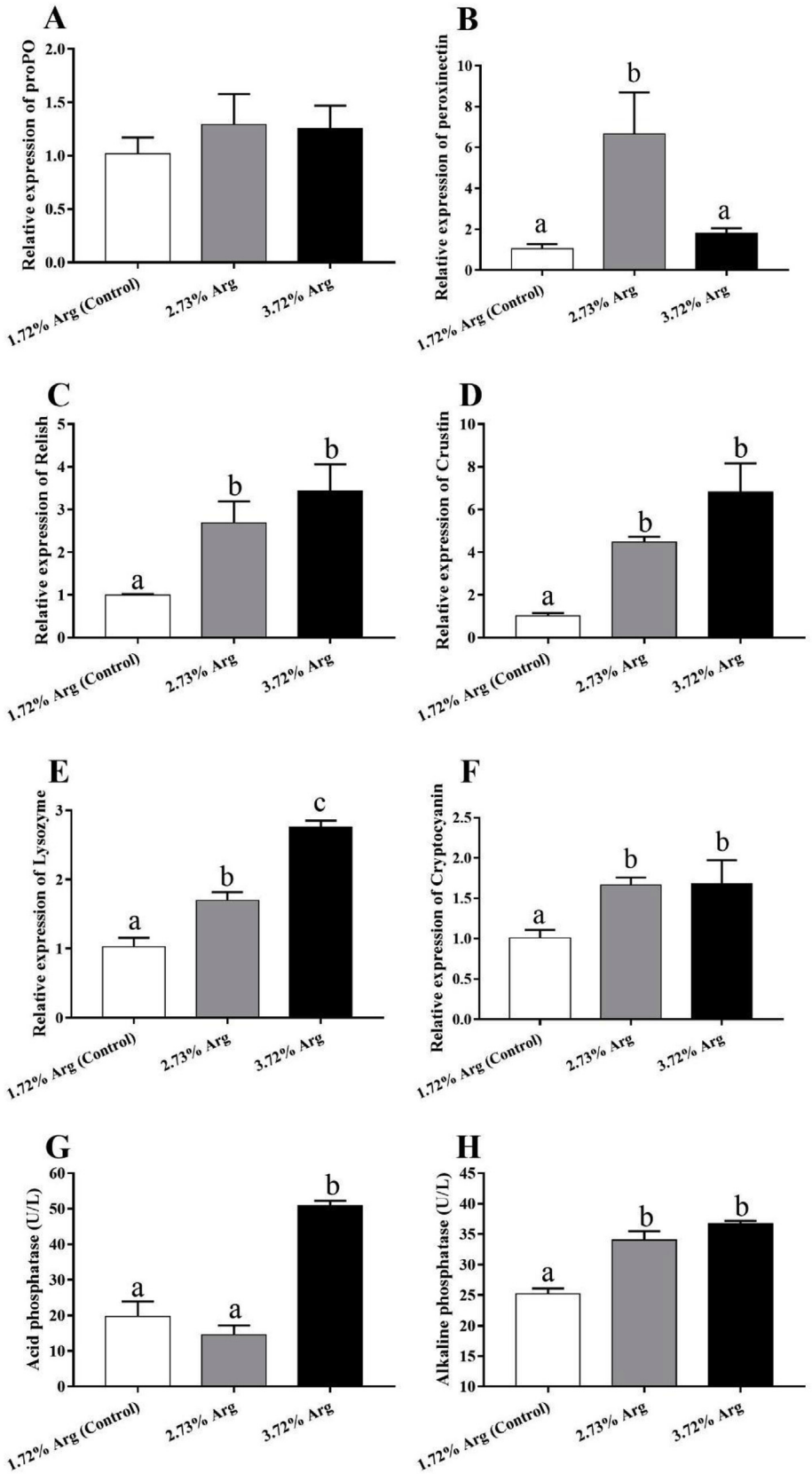

Fig. 3. Effects of arginine on the immunity of Chinese mitten crab. (A) Relative expression of proPO in the hepatopancreas, (B) Relative expression of peroxinectin in the hepatopancreas, (C) Relative expression of relish in the hepatopancreas, (D) Relative expression of Crustin in the hepatopancreas, (E) Relative expression of lysozyme in the hepatopancreas, (F) Relative expression of cryptocyanin in the hepatopancreas. (G) Acid phosphatase in the hemolymph, (H) Alkaline phosphatase in the hemolymph, Different lowercase letters show significant differences ($P < 0.05$).

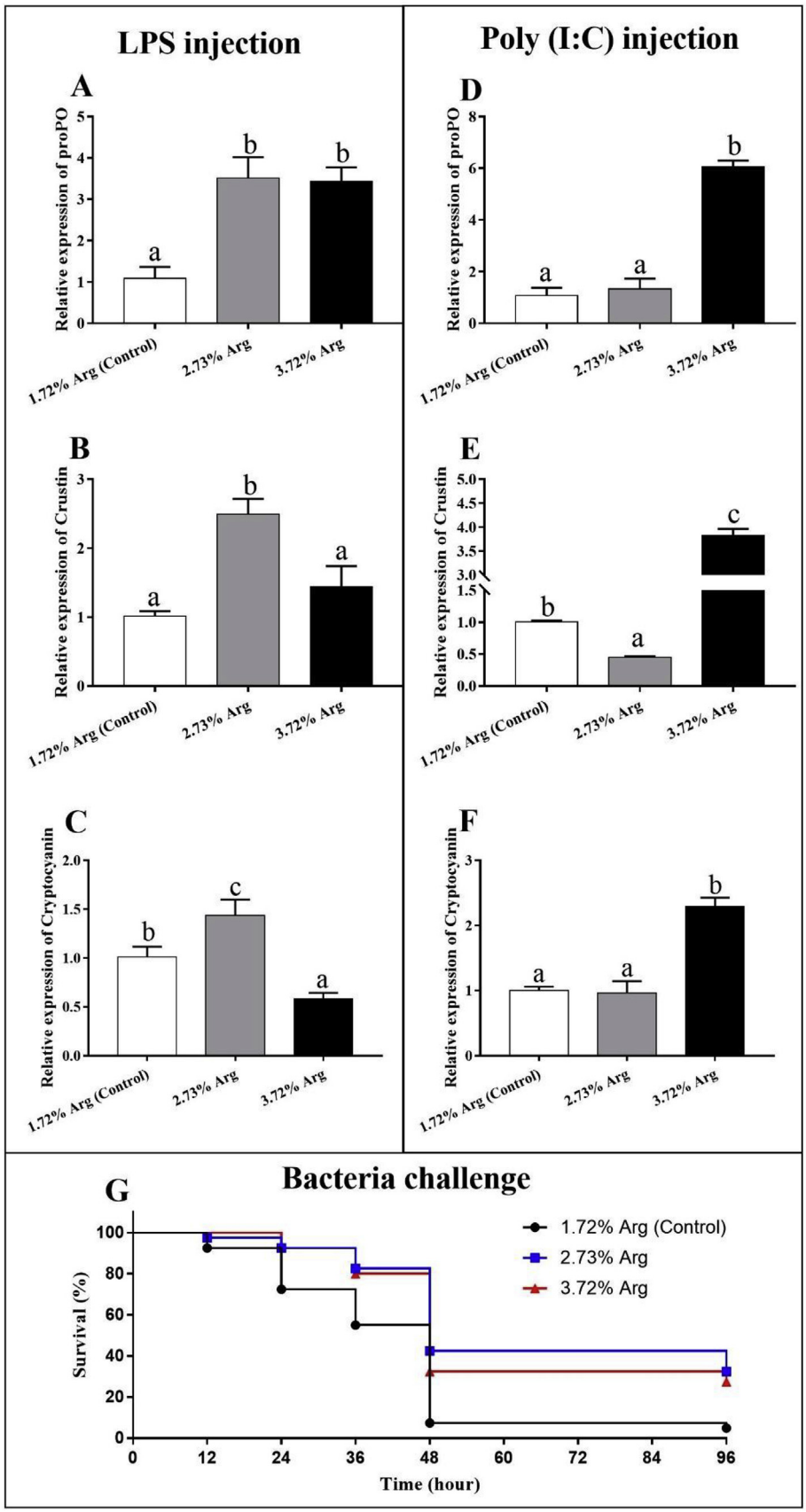

Fig. 4. Effects of arginine on the disease resistance of Chinese mitten crab. (A) Relative expression of proPO after LPS injection, (B) Relative expression of crustin after LPS injection, (C) Relative expression of cryptocyanin after LPS injection, (D) Relative expression of proPO after Poly (I:C) injection, (E) Relative expression of crustin after Poly (I:C) injection, (F) Relative expression of cryptocyanin after Poly (I:C) injection, (G) *Aeromonas hydrophila* resistance of Chinese mitten crab. Different lowercase letters show significant differences ($P < 0.05$).

4. Discussion

In the present study, dietary supplementation with Arg improved the weight gain of Chinese mitten crab, which is similar to the results reported on this species [15] and other crustaceans such as Pacific white shrimp (*Litopenaeus vannamei*) [13], Atlantic ditch shrimp (*Palaemonetes varians*) [37] and Kuruma shrimp (*Marsupenaeus japonicus*) [38]. It is well known that the growth of animals is regulated by growth factors and Arg can serve as a potent stimulant of insulin [8] and growth hormone [9] to regulate animal growth. In the present study, Arg increased the contents of ecdysterone and growth hormone, and upregulated the relative expression of IGF2 gene in the Arg supplementation groups, which is similar to the results reported on the Pacific salmon, rainbow trout [8] and goldfish [9]. Growth occurs mainly due to protein and lipid deposition in the body and Arg contributes to growth as an amino acid to participating protein synthesis [39–42]. In the present study, the whole-body protein contents in the Arg supplementation groups were higher than that in the control, which is similar to the result reported in juvenile hybrid sturgeon (*Acipenser schrenckii* ♀ × *A. baerii* ♂) [43] and juvenile cobia (*Rachycentron canadum*) [40]. This result indicates that Arg is able to promote protein synthesis and deposition in Chinese mitten crab. An interesting finding in this study is that crude lipid in the whole body increased with the increasing level of Arg supplementation in diets, which was also found in fish such as juvenile cobia (*R. canadum*) [40]. The underlying mechanism is possibly because Arg can enhance lipogenesis by increasing the PPARγ expression in the preadipocytes [44,45]. However, these results are different from other studies in Pacific white shrimp [13] and Kuruma shrimp [38], where lipid was not affected by dietary Arg level. This discrepancy may due to a species-specific reason, and the mechanism warrants further study.

In the present study, the survival of crabs was significantly lower in the Arg deficient group. The similar result was obtained in crab at the megalopae stage [14]. Survival is related to the antioxidant capacity of organisms, and when the crab is in an unhealthy state, oxidative stress can occur and result in a higher mortality [46]. Our results show that the MDA content was significantly higher in the Arg deficient group, suggesting that Arg deficiency enhances lipid peroxidation in the hepatopancreas of a crab. This may be one of the possible reasons for the higher mortality in the Arg deficient group. Arg has the ability to enhance the antioxidant capacity and reduce the oxidative stress in aquatic animals [16,18]. In the antioxidative system, SOD is an important enzyme to reduce the stress of superoxide anions [47,48]. The end product of the dismutation reaction, H_2O_2, can be removed by the catalase [48]. In the present study, Arg increased the activities of SOD and CAT, which is similar to the studies in juvenile blunt snout bream (*Megalobrama amblycephala*) [16] and Jian carp (*C. carpio* var. Jian) [18]. Furthermore, the activity of antioxidant enzymes is regulated by the Keap1/Nrf2 signaling pathway [18], where KEAP1 is a specialized sensor to quantify stress by monitoring the intracellular concentrations of NO, Zn^{2+}, and alkenals [49]. Our study found that Arg increased the expression of KEAP1 to allow crab to perceive stress response and regulate the antioxidant enzymes. Our study indicates that Arg is able to alleviate oxidative stress via increasing antioxidative enzymes such as SOD and CAT in Chinese mitten crab.

In addition, Arg can improve nitric oxide (NO) production [50,51] to regulate antioxidant capacity through the AMPK-NO signaling pathway [16]. Besides, NO can also form NOO- free radicals to induce toxic effect on an organism. Studies in juvenile blunt snout bream found that excessive NO could inhibit antioxidant enzymes [52], and lead to oxidative stress damage and inflammation [16]. Our study found that the nitric oxide (NO) content in the hemolymph was high in the Arg deficient group. It can be speculated that the NO content in hemolymph may be excessive, and a high NO content may cause oxidative stress in Chinese mitten crab when dietary Arg cannot satisfy the requirement. When Arg was supplemented into the diets, the NO content decreased. Possibly, Arg can reduce the NO content of the crab under the oxidative stress, which is similar to the study in mammals [53].

Arg also plays an important role in immune responses by Arg metabolism [4]. In the present study, Arg supplementation increased the activities of ACP and AKP, which are the first line innate immunity defense enzymes [54], and are an important immune index in shrimp and crab. Except for the immune enzymes, immune factors in the hemolymph also play an important role in the immune system of crustacean [55]. Cryptocyanin is a critical immune factor in the hemolymph of crustacean [55], and can resist the infections of white spot syndrome virus by up-regulation of cryptocyanin expression in mud crab (*Scylla serrata*) [56]. In the present study, the relative expressions of cryptocyanin were significantly upregulated in the Arg supplementation groups. It indicates that Arg can enhance the cryptocyanin in the hemolymph to improve the immunity and disease resistance of Chinese mitten crab.

Crabs have no adaptive immunity and largely rely on innate immunity including cellular and humoral immunity to defend invading microbes [21,57,58]. Peroxinectin is one of the cell adhesion factors in the innate immunity system with the features of both peroxidase activity and adhesive property in crustaceans [59], and can be activated after the challenge of pathogens [24]. In the present study, the relative expression of peroxinectin was upregulated in crabs fed the 2.73% Arg diet. Furthermore, peroxinectin activation is associated with proPO system [24], which is one of the important components of innate immunity only found in invertebrates [22]. A previous study reported that proPO is potentially involved in the acute response against invading bacteria in Chinese mitten crab [22]. In the present study, although Arg did not upregulate the gene expression of proPO, the slightly higher expressions were detected in the Arg supplementation groups. We speculate that Arg could upregulate the gene expression of peroxinectin and the increased proPO expression would enhance the immunity and disease resistance in Chinese mitten crab. In addition, peroxinectin is a homologue of MPO [24], and can enhance the nuclear translocation of the nuclear factor NF-kB [60]. Furthermore, activation and translocation of NF-kB are associated with antibacterial peptide expression in invertebrates [61]. Therefore, we tested the gene expressions of relish, a NF-κB-like transcription factor [62], and antibacterial peptide genes including crustin and lysozyme. The results showed that relish, crustin and lysozyme could be significantly upregulated in the Arg supplementation diets. Therefore, Arg has the ability to enhance the immunity of Chinese mitten crab through the peroxinectin-relish-crustin/Lysozyme pathway.

In order to test whether Arg can really increase the ability to resist bacterial and viral infections in crab, the LPS and Poly (I:C) were used to perform stimulation tests as LPS is a bacterial analog and Poly (I:C) is a viral analogues. After LPS and Poly (I:C) stimulations, the gene expressions of crustin, cryptocyanin and proPO were significantly upregulated in the 2.73% Arg and 3.72% Arg diets, respectively. These results indicate that the diet containing 2.73% Arg may enhance bacteria resistance and 3.72% Arg may improve anti-virus capability in Chinese mitten crab by upregulating the genes involved in the immune response. In the mosquito *Anopheles stephensi*, Arg is involved in the immune regulation through the NOS pathway as it can reduce the infection of plasmodium parasites by increasing the synthesis of nitric oxide [25]. Other studies on spiny lobster (*Panulirus argus*) [63,64], shrimp (*Marsupenaeus japonicus*) [65], crayfish (*Procambarus clarkii*) [66] and mud crab [67] also showed that the NOS activity is associated with the resistance of pathogen infection. In the present study, the NO content in the hemolymph and the activities of TNOS and iNOS in the hepatopancreas were higher in the control diet. In contrast, the NO content and the activities of TNOS and iNOS decreased in crabs fed the Arg supplementation diets, suggesting that the immune regulation in crabs may not go through the NO pathway, which leaves future research to confirm.

Finally, crabs fed diets containing 2.73% and 3.72% Arg had a

higher survival after *Aeromonas hydrophila* injection, which is similar to a previous study on *Litopenaeus vannamei* [68]. The present study suggests that Arg is an important amino acid to enhance the ability of disease resistance in Chinese mitten crab.

5. Conclusion

Dietary supplementation with 2.73% and 3.72% arginine promoted weight gain, crude protein and crude lipid contents probably via up-regulating IGF2 and increasing the contents of ecdysterone, growth hormone. Furthermore, Arg supplementation with 2.73% and 3.72% arginine also improved the antioxidant capacity and immunity by increasing the activities of antioxidant enzymes and the mRNA expression of genes relevant to nonspecific immunity in Chinese mitten crab.

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References

[1] K. Mai, Aquatic Animal Nutrition and Feed Science, China Agriculture Press, Beijing, China, 2011.
[2] J.E. Halver, R.W. Hardy, Fish Nutrition, Academic press, California, USA, 2002.
[3] NRC, Nutrient Requirements of Fish and Shrimp, National academies press, Washington, USA, 2011.
[4] B. Vincenzo, Z. Paola, Regulation of immune responses by L-arginine metabolism, Nat. Rev. Immunol. 5 (8) (2005) 641–654.
[5] S.H. Kim, J. Roszik, E.A. Grimm, S. Ekmekcioglu, Impact of L-arginine metabolism on immune response and anticancer immunotherapy, Front. Oncol. 8 (2018) 1–5.
[6] G. Liu, W. Ren, J. Fang, C.A. Hu, G. Guan, N.A. Al-Dhabi, J. Yin, V. Duraipandiyan, S. Chen, Y. Peng, L-Glutamine and L-arginine protect against enterotoxigenic *Escherichia coli* infection via intestinal innate immunity in mice, Amino Acids 49 (12) (2017) 1–10.
[7] B.J. Alejandro, R.B. Martha, M.D.J. Romero-Geraldo, F.D.J. Ascencio-Valle, Effects of dietary arginine on hematological parameters and innate immune function of channel catfish, J. Aquat. Anim. Health 19 (3) (2007) 195–203.
[8] E.M. Plisetskaya, L.I. Buchelli-Narvaez, R.W. Hardy, W.W. Dickhoff, Effects of injected and dietary arginine on plasma insulin levels and growth of pacific salmon and rainbow trout, Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 98 (1) (1991) 165–170.
[9] A.D. Uretsky, J.P. Chang, Evidence that nitric oxide is involved in the regulation of growth hormone secretion in goldfish, Gen. Comp. Endocrinol. 118 (3) (2000) 461–470.
[10] J.L. Wan, K.S. Mai, Q.H. Ai, The recent advance on arginine nutritional physiology in fish, J. Fish. Sci. China/Zhongguo Shuichan Kexue 13 (4) (2006) 679–685.
[11] Z. Cheng, D.M.G. Iii, A. Buentello, Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), Aquaculture 362–363 (2012) 39–43.
[12] H. Lin, X. Tan, C. Zhou, J. Niu, D. Xia, Z. Huang, J. Wang, Y. Wang, Effect of dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano *Trachinotus ovatus*, Aquaculture 437 (2015) 382–389.
[13] Q.C. Zhou, W.P. Zeng, H.L. Wang, T. Wang, Y.L. Wang, F.J. Xie, Dietary arginine requirement of juvenile Pacific white shrimp, *Litopenaeus vannamei*, Aquaculture 364–365 (1) (2012) 252–258.
[14] H.B. Jiang, K. Li, L.Q. Chen, Q. Wang, Q. Liu, L. Liu, Nutritional requirement of the Chinese mitten-handed crab *Eriocheir sinensis* juvenile for arginine and lysine, J. World Aquac. Soc. 36 (4) (2010) 515–520.
[15] J.Y. Ye, Y.H. Wang, J.L. Guo, J.M. Chen, Q. Pan, B.Q. Shen, Lysine,methionine and arginine requirements of juvenile Chinese mitten crab (*Eriocheir sinensis*), J. Fish. China 34 (10) (2010) 1541–1548.
[16] H. Liang, K. Ji, X. Ge, M. Ren, B. Liu, B. Xi, L. Pan, Effects of dietary arginine on antioxidant status and immunity involved in AMPK-NO signaling pathway in juvenile blunt snout bream, Fish Shellfish Immunol. 78 (2018) 69–78.
[17] B. Tan, Y.L. Yin, X.F. Kong, P. Li, X.L. Li, H.J. Gao, X.G. Li, R.L. Huang, G.Y. Wu, L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells, Amino Acids 38 (4) (2010) 1227–1235.
[18] B. Wang, L. Feng, G.F. Chen, W.D. Jiang, Y. Liu, S.Y. Kuang, J. Jiang, L. Tang, P. Wu, W.N. Tang, Y.A. Zhang, J. Zhao, X.Q. Zhou, Jian carp (*Cyprinus carpio* var. Jian) intestinal immune responses, antioxidant status and tight junction protein mRNA expression are modulated via Nrf2 and PKC in response to dietary arginine deficiency, Fish Shellfish Immunol. 51 (2016) 116–124.
[19] G. Chen, Y. Liu, J. Jiang, W. Jiang, S. Kuang, L. Tang, W. Tang, Y.A. Zhang, X. Zhou, L. Feng, Effect of dietary arginine on the immune response and gene expression in head kidney and spleen following infection of Jian carp with *Aeromonas hydrophila*, Fish Shellfish Immunol. 44 (1) (2015) 195–202.
[20] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, R. Ezekowitz, Phylogenetic perspectives in innate immunity, Science 284 (5418) (1999) 1313–1318.
[21] C. Mu, P. Zheng, J. Zhao, L. Wang, L. Qiu, H. Zhang, Y. Gai, L. Song, A novel type III crustin (*CrusEs 2*) identified from Chinese mitten crab *Eriocheir sinensis*, Fish Shellfish Immunol. 31 (1) (2011) 142–147.
[22] Y. Gai, J. Zhao, L. Song, C. Li, P. Zheng, L. Qiu, D. Ni, A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: gene cloning, expression and activity analysis, Fish Shellfish Immunol. 24 (2) (2008) 156–167.
[23] M.W. Johansson, K. Söderhäll, Isolation and purification of a cell adhesion factor from crayfish blood cells, J. Cell Biol. 106 (5) (1988) 1795–1803.
[24] X. Lin, L. Cerenius, B.L. Lee, K. Söderhäll, Purification of properoxinectin, a myeloperoxidase homologue and its activation to a cell adhesion molecule, Biochim. Biophys. Acta 1770 (1) (2007) 87–93.
[25] S. Luckhart, Y. Vodovotz, L. Cui, R. Rosenberg, The mosquito Anopheles stephensi limits malaria parasite development with inducible synthesis of nitric oxide, Proc. Natl. Acad. Sci. U.S.A. 95 (10) (1998) 5700–5705.
[26] S. Wang, Y. He, Y. Wang, N. Tao, X. Wu, X. Wang, W. Qiu, M. Ma, Comparison of flavour qualities of three sourced *Eriocheir sinensis*, Food Chem. 200 (2016) 24–31.
[27] B.o.f.a.f.m, Ministry of Agriculture and Rural Affairs, N.F.T.E.C. China Society of Fisheries, China Fishery Statistical Yearbook, China Agriculture Press, Beijing, China, 2018.
[28] Z. Ding, Q. Meng, H. Liu, S. Yuan, F. Zhang, M. Sun, Y. Zhao, M. Shen, G. Zhou, J. Pan, First case of hepatopancreatic necrosis disease in pond-reared Chinese mitten crab, *Eriocheir sinensis*, associated with microsporidian, J. Fish Dis. 39 (9) (2016) 1043–1051.
[29] G. Chen, X. Wang, A review of common diseases of mitten crab, Aquaculture (China) 33 (3) (2012) 46–48.
[30] F. Han, X. Wang, J. Guo, C. Qi, C. Xu, Y. Luo, E. Li, J.G. Qin, L. Chen, Effects of glycinin and β-conglycinin on growth performance and intestinal health in juvenile Chinese mitten crabs (*Eriocheir sinensis*), Fish Shellfish Immunol. 84 (2019) 269–279.
[31] X. Jin, W. Li, C. Lin, L. Shuang, X. Guo, A. Yu, M. Wu, L. He, Q. Wang, Two novel short C-type lectin from Chinese mitten crab, *Eriocheir sinensis*, are induced in response to LPS challenged, Fish Shellfish Immunol. 33 (5) (2012) 1149–1158.
[32] X. Du, Y. Li, D. Li, F. Lian, S. Yang, J. Wu, H. Liu, G. Bu, F. Meng, X. Cao, Transcriptome profiling of spleen provides insights into the antiviral mechanism in *Schizothorax prenanti* after poly (I: C) challenge, Fish Shellfish Immunol. 62 (2017) 13–23.
[33] S. Sun, J. Qin, N. Yu, X. Ge, H. Jiang, L. Chen, Effect of dietary copper on the growth performance, non-specific immunity and resistance to *Aeromonas hydrophila* of juvenile Chinese mitten crab, *Eriocheir sinensis*, Fish Shellfish Immunol. 34 (5) (2013) 1195–1201.
[34] AOAC, Official Methods of Analysis of AOAC International, eighteenth ed., Association of Official Analytical Chemists, Washington DC, USA, 2005.
[35] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) Method, Methods 25 (4) (2001) 402–408.
[36] J. Liu, J. Wang, W. Yue, J. Chen, S. Huang, Y. Ci, C. Wang, The effect of pH on the molting,growth and related gene expression in juvenile mitten crab, *Eriocheir sinensis*, Freshw. Fish. 46 (4) (2016) 96–100.
[37] J. Palma, J.P. Andrade, A. Lemme, D.P. Bureau, Quantitative dietary requirement of juvenile Atlantic ditch shrimp *Palaemonetes varians* for lysine, methionine and arginine, Aquacult. Res. 46 (8) (2015) 1822–1830.
[38] M.S. Alam, S.I. Teshima, M. Ishikawa, D. Hasegawa, S. Koshio, Dietary arginine requirement of juvenile kuruma shrimp *Marsupenaeus japonicus* (Bate), Aquacult. Res. 35 (9) (2004) 842–849.
[39] G. Wu, S.J. Morris, Arginine metabolism: nitric oxide and beyond, Biochem. J. 336 (1) (1998) 1–17.
[40] M. Ren, Q. Ai, K. Mai, Dietary arginine requirement of juvenile cobia (*Rachycentron canadum*), Aquacult. Res. 45 (2) (2014) 225–233.
[41] K. Yao, Y.L. Yin, W. Chu, Z. Liu, D. Deng, T. Li, R. Huang, J. Zhang, B. Tan, W. Wang, Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs, J. Nutr. 138 (5) (2008) 867–872.
[42] W.J. Jobgen, C.J. Meininger, S.C. Jobgen, P. Li, M.J. Lee, S.B. Smith, T.E. Spencer, S.K. Fried, G.Y. Wu, Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats, J. Nutr. 139 (2) (2009) 230–237.
[43] L. Wang, J. Wu, C.a. Wang, J. Li, Z. Zhao, L. Luo, X. Du, Q. Xu, Dietary arginine requirement of juvenile hybrid sturgeon (*Acipenser schrenckii* ♀ × *Acipenser baerii* ♂), Aquacult. Res. 48 (10) (2017) 5193–5201.
[44] H.Y. Yan, A. Edith, S. Gillian, W. Alex, S. Deena, K. Abdenaim, A.H. Mohammed, D.C.W. Lau, Nitric oxide promotes differentiation of rat white preadipocytes in culture, J. Lipid Res. 43 (12) (2002) 2123–2129.
[45] S.T. Ding, A.P. Schinckel, T.E. Weber, H.J. Mersmann, Expression of porcine transcription factors and genes related to fatty acid metabolism in different tissues and genetic populations, J. Anim. Sci. 78 (8) (2000) 2127–2134.
[46] Z. Wang, C.F. Cai, X.M. Cao, J.M. Zhu, J. He, P. Wu, Y.T. Ye, Supplementation of dietary astaxanthin alleviated oxidative damage induced by chronic high pH stress, and enhanced carapace astaxanthin concentration of Chinese mitten crab *Eriocheir sinensis*, Aquaculture 483 (2018) 230–237.
[47] I. Dar, A. Barzilai, Cellular Responses to Oxidative Stress, Springer, Netherlands, 2009.

[48] L. Canesi, Pro-oxidant and antioxidant processes in aquatic invertebrates, Ann. N. Y. Acad. Sci. 1340 (1) (2014) 1–7.
[49] M.M. Michael, D.J. Lamont, K.A. Beattie, J.D. Hayes, Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals, Proc. Natl. Acad. Sci. U.S.A. 107 (44) (2010) 18838–18843.
[50] L. Zheng, Y. Pan, Y. Feng, L. Cui, Y. Cao, L-Arginine supplementation in mice enhances NO production in spleen cells and inhibits *Plasmodium yoelii* transmission in mosquitoes, Parasit. Vector 8 (1) (2015) 1–6.
[51] Y. Tu, S. Xie, H. Dong, Y. Yang, J. Jin, X. Zhu, Dietary arginine requirement for gibel carp (*Carassis auratus gibelio* var. CAS III) reduces with fish size from 50 g to 150 g associated with modulation of genes involved in TOR signaling pathway, Aquaculture 449 (2015) 37–47.
[52] T.S. Kostic, S.A. Andric, D. Maric, R.Z. Kovacevic, Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis, J. Steroid Biochem. Mol. Biol. 75 (4) (2000) 299–306.
[53] G. Yue, J. Yan, C. Li, Effects of exogenous NO precursor L-arg on myocardium in rat after one-off exhaustive swimming training, J. Xi'an Phy. Educ. Univ. 25 (1) (2008) 81–85.
[54] S.M. Limbu, L. Zhou, S.X. Sun, M.L. Zhang, Z.Y. Du, Chronic exposure to low environmental concentrations and legal aquaculture doses of antibiotics cause systemic adverse effects in Nile tilapia and provoke differential human health risk, Environ. Int. 115 (2018) 205–219.
[55] X.Q. Wang, L.L. Wang, M.Q. Wang, H. Zhang, L.S. Song, CDNA cloning, characterization and mRNA expression of cryptocyanin from the Chinese mitten crab, Eriocheir sinensis H. Milne Edwards, 1853, Crustaceana 89 (3) (2016) 273–290.
[56] L. Wen, Q. Dong, X. Yan, Proteomic analysis of differentially expressed proteins in hemolymph of *Scylla serrata* response to white spot syndrome virus infection, Aquaculture 314 (1) (2011) 53–57.
[57] M. Divya, B. Vaseeharan, M. Anjugam, A. Iswarya, S. Karthikeyan, P. Velusamy, M. Govindarajan, N.S. Alharbi, S. Kadaikunnan, J.M. Khaled, Phenoloxidase activation, antimicrobial, and antibiofilm properties of β-glucan binding protein from *Scylla serrata* crab hemolymph, Int. J. Biol. Macromol. 114 (2018) 864–873.
[58] Y.S. Kim, J.H. Ryu, S.J. Han, K.H. Choi, K.B. Nam, I.H. Jang, B. Lemaitre, P.T. Brey, W.J. Lee, Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells, J. Biol. Chem. 275 (42) (2000) 32721–32727.
[59] S. Lv, B. Lu, J. Xu, H. Xu, J. Zhao, S. Li, Y. Li, Y. Chen, Immune response of peroxinectin of Chinese mitten crab *Eriocheir sinensis* to exterior stimulation, Dev. Comp. Immunol. 51 (1) (2015) 56–64.
[60] L. Denise, M. Hanke, J.P. Eiserich, B.A. Freeman, D. Andreas, U.M. Gehling, B. Jens, R. Volker, M. Thomas, H. Thomas, Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins, Proc. Natl. Acad. Sci. U.S.A. 102 (2) (2005) 431–436.
[61] J.A. Hoffmann, The immune response of *Drosophila*, Nature 426 (6962) (2003) 33–38.
[62] F. Li, L. Wang, H. Zhang, P. Zheng, J. Zhao, L. Qiu, Y. Zhang, L. Song, Molecular cloning and expression of a Relish gene in Chinese mitten crab *Eriocheir sinensis*, Int. J. Immunogenet. 37 (6) (2010) 499–508.
[63] T. Rodríguez-Ramos, Y. Carpio, J. Bolívar, G. Espinosa, J. Hernández-López, T. Gollas-Galván, L. Ramos, C. Pendón, M.P. Estrada, An inducible nitric oxide synthase (NOS) is expressed in hemocytes of the spiny lobster *Panulirus argus*: cloning, characterization and expression analysis, Fish Shellfish Immunol. 29 (3) (2010) 469–479.
[64] T. Rodríguez-Ramos, Y. Carpio, L. Ramos, T. Pons, O. Farnós, C. Iglesias, I.S.D. Melo, Y. Ramos, C. Pendón, M.P. Estrada, New aspects concerning to the characterization and the relationship with the immune response in vivo of the spiny lobster *Panulirus argus* nitric oxide synthase, Nitric Oxide 25 (4) (2011) 396–406.
[65] G. Jiang, R. Yu, M. Zhou, Studies on nitric oxide synthase activity in haemocytes of shrimps *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* after white spot syndrome virus infection, Nitric Oxide 14 (3) (2006) 219–227.
[66] F.C. Yeh, S.H. Wu, C.Y. Lai, C.Y. Lee, Demonstration of nitric oxide synthase activity in crustacean hemocytes and anti-microbial activity of hemocyte-derived nitric oxide, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 144 (1) (2006) 11–17.
[67] S. Li, Z. Zhang, C. Li, L. Zhou, W. Liu, Y. Li, Y. Zhang, H. Zheng, X. Wen, Molecular cloning and expression profiles of nitric oxide synthase (NOS) in mud crab *Scylla paramamosain*, Fish Shellfish Immunol. 32 (4) (2012) 503–512.
[68] C.H. Zhu, L. Li, T. Wu, S. Shi, Z. Wu, Effects of L-arginine on the humoral immuning factors of *Litopenaeus vannamei*, Mar. Sci. 33 (2) (2009) 55–59.