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Effect of N-carbamoylglutamate supplementation on the growth performance, antioxidant status and immune response of mirror carp (*Cyprinus carpio*) fed an arginine-deficient diet

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ABSTRACT

The present study was conducted to determine the effect of dietary N-carbamoylglutamate (NCG) supplementation on the growth performance, antioxidant capability and immune responses of mirror carp (*Cyprinus carpio*) fed an arginine (Arg)-deficient diet. A total of 630 mirror carp (41.65 ± 0.14 g) were fed diets (Arg 1.24% of the diet) that were supplemented with 0.50% Arg (control diet) or graded levels of NCG at 0 (Arg deficiency diet), 0.04%, 0.08%, 0.12%, 0.16% and 0.20% for 8 weeks. The results showed that, compared with the control diet, the Arg-deficient diet supplementation with 0 NCG (1) decreased the final body weight (FWB), the weight gain rate (WGR) or the protein efficiency ratio (PER) and increased the feed conversion ratio (FCR); (2) decreased the concentration of Arg and nitric oxide (NO) and the activity of total nitric oxide synthetase (T-NOS) in the plasma; (3) decreased the activities of superoxide dismutase (SOD) in the proximal intestine (PI), catalase (CAT) in the PI and distal intestine (DI), and glutathione peroxidase (GPx) in PI and mid-intestine (MI) and increased the concentration of malondialdehyde (MDA) in the PI, MI and DI; and (4) decreased the activity of lysozyme in the plasma, increased the relative mRNA expression of tumor necrosis factor-α (TNF-α), interleukin1β (IL-1β) and interleukin 8 (IL-8) in the PI, MI and DI, and decreased the relative mRNA expression of interleukin 10 (IL-10) in the PI and MI, and transforming growth factor β2 (TGF-β2) in the PI, MI and DI. Compared with the Arg deficient-diet supplementation with 0 NCG, (1) 0.12% or 0.16% NCG increased the FBW, WGR and PER, and 0.16% NCG increased the FCR; (2) 0.08%–0.20% NCG increased the concentration of Arg, NO and the activity of T-NOS; (3) 0.08% NCG increased the activities of SOD in the PI and MI, and 0.12% NCG increased activities of CAT and GPx in the PI, MI and DI; and (4) 0.04%–0.20% NCG increased the activity of lysozyme, 0.04%–0.20% NCG decreased the relative mRNA expression of TNF-α, IL-1β and IL-8 in the PI and MI, and 0.04%–0.20% NCG increased the relative mRNA expression of IL-10 and TGF-β2 in the PI and MI. The present results indicated that dietary 0.12% or 0.16% NCG improved the growth performance, feed utilization, intestinal antioxidant capacity and immune response of mirror carp fed an Arg-deficient diet.

1. Introduction

Proteins and amino acids are critical molecules because of the role they play in the structure and metabolism of all living organisms. Arginine (Arg) is one of ten essential amino acids in all fish species [1]. Arg is not only a building block for proteins, but also an essential precursor for the synthesis of biologically important metabolites, including NO, polyamines, creatine, ornithine, proline and glutamate [2,3]. In addition, Arg is a major reserve of high-energy phosphate for adenosine triphosphate (ATP) regeneration in the muscle [4]. Arg serves as a potent stimulant of insulin, insulin-like growth factor I (IGF-I) and growth hormone (GH), possibly due to its roles in anabolic processes [5] [6], [7]. The dietary Arg requirements are estimated for some fish, ranging from 1.6% to 3.1% of the diet [8]. Our previous study indicated that based on the specific growth rate (SGR), the optimal dietary Arg requirement for juvenile hybrid sturgeon (*Acipenser schrenckii* ♀ × *A. baerii* ♂) was estimated to be 2.47% of the diet [7]. Dietary Arg deficiency reduces growth and protein deposition. However, supplementation with Arg improves the growth performance in fish [6] [9], [10], [11]. Moreover, Arg is the most limiting amino acid in plant protein sources, such as corn, sesame and zein meal [12], [13]. The price of Arg is high, and thus, the feeding of crystalline Arg appears to be uneconomical.

Alternatively, N-carbamylglutamate (NCG), a metabolically stable

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Table 1
Ingredient content and approximate composition of the diets (as-fed basis).

Ingredients	Control	NCG				
		0	0.04%	0.08%	0.12%	0.16%	0.20%
Fish meal[a]	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Corn gluten meal[a]	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Soybean meal[a]	21.67	21.67	21.67	21.67	21.67	21.67	21.67
Wheat middling[b]	38.00	38.00	38.00	38.00	38.00	38.00	38.00
Corn Starch	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Soybean oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Soybean phospholipids	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Monocalcium phosphate	2.47	2.47	2.47	2.47	2.47	2.47	2.47
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Sodium carboxymethylcellulose	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[c]	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin premix[d]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Methionine	0.66	0.66	0.66	0.66	0.66	0.66	0.66
Cellulose	0	0.50	0.46	0.42	0.38	0.34	0.30
NCG	0	0	0.04	0.08	0.12	0.16	0.20
Arg	0.5	0	0	0	0	0	0
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition (%)[e]							
Crude protein	32.65	32.53	32.32	32.68	32.46	32.33	32.63
Crude fat	7.41	7.36	7.36	7.29	7.27	7.33	7.39
Arginine	1.79	1.24	1.22	1.26	1.24	1.23	1.24

[a] The fish meal, soya bean meal and corn gluten meal were obtained from the Weierhao Group (Harbin, China).
[b] The wheat middling was obtained from the Xiangxue Group (Shenyang, China).
[c] The mineral premix included the following (mg/kg diet): Fe, 25.00; Cu, 3.00; Mn, 15.00; Zn, 60.00; Se, 0.30; I, 0.60; and Mg, 500.00.
[d] The vitamin premix included the following: VE, 60 mg/kg; VK, 5 mg/kg; VA, 15 000 IU/kg; VD_3, 3000 IU/kg; VB_1, 15 mg/kg; VB_2, 30 mg/kg; VB_6, 15 mg/kg; VB_{12}, 0.5 mg/kg; nicotinic acid, 175 mg/kg; folic acid, 5 mg/kg; inositol, 1000 mg/kg; pantothenic acid, 50 mg/kg; biotin, 2.50 mg/kg; and VC, 125 mg/kg.
[e] Crude protein, crude fat and Arginine were measured.

analog of N-acetylglutamate (NAG), is proven to increase the endogenous synthesis of Arg and the plasma concentration of Arg by activating intestinal pyrroline-5-carboxylate synthase and carbamylphosphate synthase-1 (the rate-limiting enzymes responsible for both the urea cycle and the Arg synthetic pathway) [3], [14]. Dietary NCG supplementation increases the endogenous synthesis of Arg, the gut integrity, health and mucosal immunity, muscle protein synthesis and growth performance in piglets [3] [15], [16], [17]. In addition, dietary supplementation with NCG during gestation significantly improved the reproductive performance of sows [18] [19] [20], and rats [21], which may be associated with the improved concentrations of Arg in the plasma. Cheng et al. (2015) reported that dietary supplementation with NCG improved the weight gain and the protein efficiency ratio in Nile tilapia fed with a normal Arg content in the feed [22]. However, no study has investigated the effects of NCG on the growth performance of mirror carp (*Cyprinus carpio*) fed an Arg-deficient diet.

The intestinal mucosal, as the primary barrier between the external environment and the closely regulated internal milieu, is essential for fish, and its immunity plays an important role in protecting fish from infection [23]. Therefore, maintaining a well-functioning intestinal mucosal immune system is of vital importance for maintaining the health of fish. Arg plays a role in several immunological functions in the intestine. Evidence shows that Arg deficiency induces intestinal inflammation and decreases the intestinal antioxidant level [24]. Arg deficiency upregulates IL-1β, IL-10, and TGF-β and decreases the gene expression of TNF-α and the activities of copper/zinc SOD, GPx, CAT, glutathione reductase (GR) and glutathione-S-transferase (GST) [24]. Dietary Arg supplementation decreases the expression of pro-inflammatory cytokine genes (IL-1β, IL-6, and TNF-α) in the LPS-induced inflamed intestine [25]. In channel catfish, Arg supplemented diets increase the production of NO by macrophages, as well as the resistance to experimental *E. ictaluri* infection [26]. In mammals, dietary NCG supplementation to the IUGR in suckling lambs decreases the concentration of IL-1β in the plasma, the gene expressions of Toll-like receptor 4 (TLR-4) and nuclear transcription factor kappa-B (NF-κB), and the concentrations of protein carbonyl and MDA in the ileum and increases the expression of the tight junction proteins claudin-1 and ZO-1 and the concentration of GSH [27]. Dietary NCG supplementation has some beneficial effects on intestinal mucosal immunity in piglets challenged by E. *coli*. The ileum SIgA and IL-10 levels and the CD4% in NCG-treated piglets are higher than in no-NCG treated piglets [17]. Additionally, an important role for NCG in activating intestinal or endogenous synthesis of Arg was discovered [3]. Thus, we hypothesized that metabolic activation of endogenous Arg synthesis may provide effective strategy to augment Arg provision in mirror carp (*Cyprinus carpio*) fed an Arg-deficient diet. The common carp (*Cyprinus carpio*) is the most extensively cultured freshwater fish species in China, and the production of Common carp is 3497 967 t in 2016. Mirror carp, a variety of common carp, accounts for an increasingly larger proportion of production due to its faster growth, better disease resistance, higher meat conversion rate and near absence of scales on the body surface. Therefore, the objective of this study was to evaluate whether NCG supplementation increased Arg levels and improved the growth performance and immune responses of mirror carp (*Cyprinus carpio*) fed an Arg-deficient diet.

2. Materials and methods

2.1. Diet preparation

Seven kinds of isoprotein (32.51% crude protein) and isolipid (7.04% crude lipid) diets were formulated to meet the protein and energy requirements of mirror carp. Fish meal (6.40% crude protein), soybean meal (4.40% crude protein) and corn gluten meal (6.10% crude protein) were used as protein sources, and fish oil, soybean oil and soybean lecithin were the lipid sources. The Arg and protein requirements for common carp are recommended to be 1.8% and 32% of the diet according to the NRC [1]. The fish fed diet was supplemented with 0.50% Arg (control diet) or graded levels of NCG at 0 (Arg deficiency diet, Arg 1.24% of diet), 0.04%, 0.08%, 0.12%, 0.16% and

0.20% for 8 weeks. NCG was produced by Animore Sci. & Tech. Co., Ltd. (Beijing, China), and the purity of NCG was great than or equal to 97%. The Arg-deficient diets were fed to the mirror carp to investigate the effect of NCG supplementation on the growth performance, antioxidant capability and immune responses of the mirror carp. All the diets were individually blended in a mixer and were then homogenized after the fish oil and soybean oil were added. Then, the mixture was made into pellet (2 mm diameter), was air-dried at room temperature and was then stored at −20 °C until further use. The experimental diet formulation and approximate compositions are shown in Table 1.

2.2. Fish and experimental conditions

Mirror carp (*Cyprinus carpio*) were obtained from the Hulan Fishing Ground (Harbin, China). The fish were acclimatized to the experimental conditions in glass aquaria of 500 L and were fed the control diet three times daily for 2 weeks. Then, the mirror carp, with an average initial weight of 41.65 ± 0.14 g, were randomly assigned to 21 aquaria with an initial stocking density of 30 fish per aquarium, and each diet was fed to three randomly assigned aquaria. All the groups of fish were fed their respective diets at a rate of 4.0% body weight per day and were divided into equal portions at 8.00, 12.00 and 16.00 h. The water quality was monitored under the conditions of pH 7.5–7.8, dissolved oxygen 6.0–7.0 mg/L and ammonia nitrogen < 0.5 mg/L.

2.3. Growth performance

At the end of the 8-week feeding trial, approximately 24 h after the last feeding, the fish were counted and weighed from each replicate at end of the experiment. The weight gain rate (WGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) were determined as follows:

WGR = (final total weight-initial total weight) / initial total weight × 100%,
FCR = dry feed intake (g) / wet weight gain (g),
PER = (weight gain (g) / protein intake (g)) × 100%.

2.4. Sample collection

All the experimental fish were anesthetized with tricane methane sulfonate MS-222 (75 mg/L) before sampling. Three fish from each tank were randomly selected, and blood samples were collected from the caudal vein using heparinized syringes. Plasma samples were obtained by centrifugation (3000 × g for 10 min) at 4 °C and were immediately stored at −20 °C until the analysis. Subsequently, the fish were killed, and the intestine was collected as three intestinal segments, including the proximal intestine (PI), mid intestine (MI), and distal intestine (DI), according to Syakuri et al. [28], which were then stored at −70 °C until the analysis.

2.5. Plasma biochemical parameters

The samples of three fish in each tank were determined. The concentration of NO and the activity of T-NOS and lysozyme in the plasma were determined according to NO or T-NOS kit protocol. All these indices were determined using commercial kits, which were supplied by Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The concentration of Arg in the plasma was analyzed by an automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan). The plasma IgM, complement 3 (C_3), and complement 4 (C_4) concentrations were determined using fish ELISA detection-kits. These three kits were purchased from the Nanjing Jiancheng Bioengineering Institute, and the measurements were conducted according to the manual, as described by Cuesta et al. [29] and Habte-tsion et al. [30].

2.6. Intestinal antioxidant status

Three intestinal tissue samples were homogenized in 10 vol (w/v) of ice-cold physiological saline solution and were centrifuged at 6000 × *g* for 20 min at 4 °C. The supernatant was then stored at −20 °C for further analysis. The supernatant was used to determine the superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activity and malondialdehyde (MDA) and soluble protein contents. All these indices were determined using commercial kits, which were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.7. Real-time quantitative PCR

Three fish from each tank were randomly selected to extract the total RNA from the PI, MI and DI. Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA) according to the manufacturer's recommended protocol. The concentration of RNA was estimated based on the absorbance at 260 nm, which was determined using a spectrophotometer. The RNA quality was determined by analysing the RNA integrity through agarose gel electrophoresis and by confirming that the A260 nm/A280 nm absorbance ratio was between 1.8 and 2.0. The expression levels of tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 1β (IL-1β), transforming growth factor β2 (TGF-β2) and β-actin were determined by quantitative real-time PCR using an ABI 7500 (ABI, USA) with a 20 μL reaction volume, containing 10 μL of 2 × SYBR® Premix Ex Taq™ (TaKaRa BIO, Tokyo, Japan), 0.8 μL of each 10 μM forward and reverse primer, 1 μL of template and 7.4 μL of sterile double-distilled water dH_2O.

The PCR specific primers were designed according to the sequences that were cloned and published in the gene bank of mirror carp for

Table 2
Primer sequences for RT-PCR analysis in this work.

Primer	Sequence Information		
	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession number
TNF-a[a]	AAGTCTCAGAACAATCAGGAA	TGCCTTGGAAGTGACATT	AJ311800
IL-1β[b]	AACTTCACACTTGAGGAT	GACAGAACAATAACAACAAC	KC008576
IL-8[c]	AAACTGAGAGTCGACGCATTG	TTTTCAATGACCTTCTTAACCCAG	EU011243.1
IL-10[d]	GCCAGCATAAAGAACTCG	CCAAATACTGCTCGATGT	JX524550.1
TGF-β2[e]	GGGACATCATCGCCATCT	TGACATTCTCGGCAGGGT	U66874.1
β-actin	GATCGGCAATGAGCGTTTCC	ACGGTGTTGGCATACAGGTC	M24113.1

[a] TNF-α, tumor necrosis factor-α.
[b] IL-1β, interleukin1β.
[c] IL-8, interleukin 8.
[d] IL-10, interleukin 10.
[e] TGF-β2, transforming growth factor β2.

Table 3
Effect of NCG on the growth and feed utilization of mirror carp (*Cyprinus carpio*) under dietary arginine deficiency.

Items	IBW (g)[a]	FBW (g)[b]	WGR (%)[c]	FCR[d]	PER[e]
Control	41.72 ± 0.43	177.11 ± 2.53[a]	324.50 ± 4.72[a]	1.26 ± 0.04[ab]	2.74 ± 0.10[ab]
0 NCG	42.11 ± 0.08	157.73 ± 3.20[b]	274.56 ± 7.58[c]	1.44 ± 0.01[a]	2.40 ± 0.02[b]
0.04% NCG	41.72 ± 0.30	159.02 ± 2.59[b]	281.26 ± 8.94 b[c]	1.42 ± 0.02[a]	2.43 ± 0.02[b]
0.08% NCG	42.12 ± 0.54	171.77 ± 6.30[ab]	307.70 ± 12.72[abc]	1.32 ± 0.09[ab]	2.64 ± 0.16[ab]
0.12% NCG	41.98 ± 0.63	173.95 ± 0.97[a]	314.50 ± 3.94[ab]	1.26 ± 0.03[ab]	2.73 ± 0.06[ab]
0.16% NCG	42.31 ± 0.28	177.00 ± 4.42[a]	318.46 ± 12.45[a]	1.22 ± 0.02[b]	2.83 ± 0.04[a]
0.20% NCG	42.38 ± 0.26	171.98 ± 7.27[ab]	306.04 ± 19.40[abc]	1.29 ± 0.10[ab]	2.70 ± 0.22[ab]

[a] IBW, initial body weight.
[b] FBW, final body weight.
[c] WGR, weight gain rate.
[d] FCR, feed conversion ratio.
[e] PER, protein efficiency ratio.

quantitative real-time PCR (Table 2). The expression values were calculated as $2^{-\Delta\Delta Ct}$ [31].

2.8. Data analysis

The data were subjected to a one-way analysis of variance (ANOVA) and are presented as the mean ± SEM using SPSS 22.0 (SPSS, Chicago, IL, USA). Differences in the means between the dietary treatments were evaluated by Duncan's multiple range tests. The level of significance was set as $P < 0.05$.

3. Results

3.1. Growth performance

The effect of NCG on the growth and feed utilization of mirror carp (*Cyprinus carpio*) under dietary Arg deficiency is shown in Table 3. Compared with the control diet, the Arg-deficient diets supplemented with 0 and 0.04% NCG decreased the FBW, WGR and PER and increased the FCR after 8 weeks of feeding ($P < 0.05$). The fish fed 0.12% or 0.16% NCG had significantly higher FWB, WGR or PER than the fish fed 0 and 0.04% NCG ($P < 0.05$). The FCR of the fish fed 0.16% NCG was lower than fish fed 0 or 0.04% NCG ($P < 0.05$). There were no significant differences in the FBW, WGR, PER or FCR between the control fish and those supplemented with 0.08%–0.20% NCG ($P > 0.05$).

3.2. The concentration of Arg and NO and the activity of T-NOS

The Arg-deficient diet supplemented with 0 NCG showed that concentration of Arg and NO and T-NOS activity in the plasma were decreased compared with the control diet ($P < 0.05$), as shown in Fig. 1. Fish fed 0.08%–0.20% NCG had a significantly increased concentration of Arg and NO compared with the 0 NCG ($P < 0.05$). The activity of T-NOS in the plasma of the fish fed 0.04%–0.20% NCG was higher than in the fish fed 0 NCG ($P < 0.05$). There were no significant differences in the concentration of Arg and NO and the activity of T-NOS in the fish fed control diet compared to the 0.08%–0.20% NCG supplemented fish ($P > 0.05$).

3.3. Intestinal antioxidant status

The effect of NCG on the activities of SOD, CAT and GPx and the concentration of MDA in the PI, MI and DI in mirror carp (*Cyprinus carpio*) under dietary Arg deficiency is shown in Fig. 2. The Arg-deficient diet supplemented with 0 NCG decreased the activities of SOD in the PI, CAT in the PI and DI, and GPx in the PI and MI compared with the control diet ($P < 0.05$). The fish fed a diet containing 0.08% NCG had significantly increased SOD activity in the PI and MI compared with the 0 NCG fish, whereas in the DI, for the 0.12% NCG group, it was significantly higher than the 0 NCG fish ($P < 0.05$). The activities of CAT and GPx in the PI, MI and DI of the fish fed 0.12% NCG was higher than the fish fed 0 NCG ($P < 0.05$). The Arg-deficient diet supplemented with 0 NCG showed an increased concentration of MDA in the PI, MI and DI compared with the control diet and the 0.12% and 0.16% NCG diets ($P < 0.05$). The results indicated that there was no significant difference in the activities of SOD, CAT and GPx and the concentration of MDA in the DI of the mirror carp between the 0 NCG and 0.20% NCG diets ($P > 0.05$).

3.4. Immune responses

As shown in Fig. 3, the Arg-deficient diet supplemented with 0 NCG showed a decreased lysozyme activity in the plasma compared with the control diet ($P < 0.05$). Fish fed 0.04%–0.20% NCG had a significantly increased lysozyme activity compared with the 0 NCG fish ($P < 0.05$). The concentrations of lgM, C3 and C4 were not significantly different among the seven groups ($P > 0.05$).

Compared with the control diet, the Arg-deficient diet supplemented with 0 NCG showed an increased relative mRNA expression for TNF-α, IL-1β and IL-8 in the PI, MI and DI ($P < 0.05$), as shown in Fig. 4. Compared with 0 NCG, the fish fed diets containing 0.04%–0.20% NCG showed significantly decreased relative mRNA expressions of TNF-α, IL-1β and IL-8 in the PI and MI, whereas in the DI, 0.08% to 0.20 NCG significantly decreased the relative mRNA expression of TNF-α, 0.04% to 0.20 NCG significantly decreased the relative mRNA expression of IL-1β, and 0.16% to 0.20 NCG significantly decreased the relative mRNA expression of IL-8. Compared with the control diet, 0 NCG decreased the relative mRNA expression of IL-10 (not in the DI) and TGF-β2 in the PI, MI and DI ($P < 0.05$). Compared with 0 NCG, 0.04%–0.20% NCG significantly increased the relative mRNA expression of IL-10 in the PI and MI, and TGF-β2 in the PI, MI and DI ($P < 0.05$).

4. Discussion

4.1. Growth performance

The Arg requirement for common carp is recommended to be 1.8% of the diet, according to Chen et al. [32], according to the NRC, the diet of Arg amount for the carp should be 1.7% [1]. In the present study, no fish died, but a poor growth performance and feed utilization were found in the fish fed with the Arg-deficient diets (1.24% of the diet) compared with the control diet (1.79% of the diet). The results of the present study were similar to the reported values for some species, such as the yellow grouper (*Epinephelus awoara*) [8], red sea bream (*Pagrus major*) [11], blunt snout bream (*Megalobrama amblycephala*) [33] [34], golden pompano (*Trachinotus ovatus*) [9], hybrid sturgeon (*Acipenser*

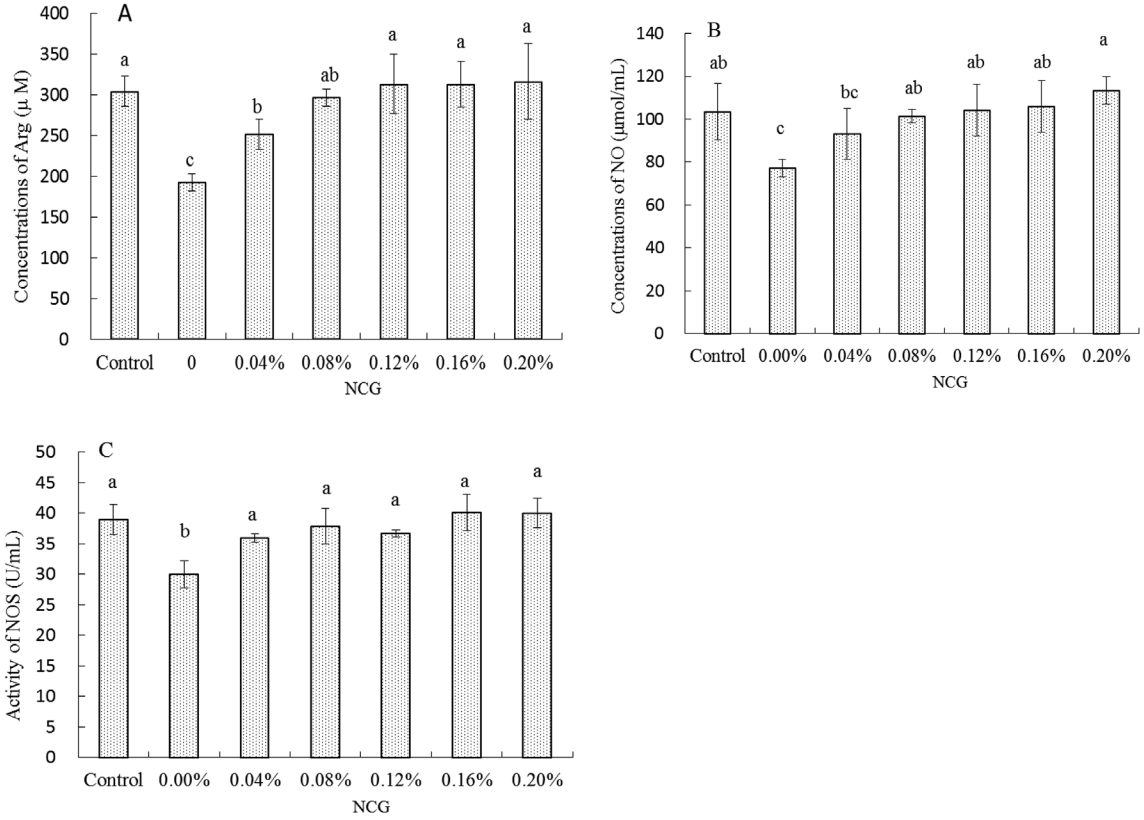

Fig. 1. Effect of NCG on the concentration of arginine (Arg) (A), nitric oxide (NO) (B) and the activity of nitric oxide synthetase (NOS) (C) in mirror carp (*Cyprinus carpio*) under dietary arginine deficiency.
The data represent the mean of three replicates. The different small letters indicate significant differences ($P < 0.05$) between the different treatments.

schrenckii × *Acipenser baerii*) [7], Japanese flounder (*Paralichthys olivaceus*) [35], Jian carp (*Cyprinus carpio var. Jian*) [32], cobia (*Rachycentron* canadum) [36], sea bream (*Sparus macrocephalus*) [37], Nile tilapia (*Oreochromis niloticus*) [38], and largemouth bass (*Micropterus salmoides*) [39]. Dietary Arg may improve growth given its role as secretagogue of growth regulating hormones (growth hormone (GH) and insulin-like growth factor I (IGF-I)) [40]. Arg participates in several growth-related processes, but studies mainly focus on its insulinotropic effect, which stimulates amino acid uptake and protein synthesis [41]. The investigated fish in these studies include pacific salmon and rainbow trout [42], [43]. Recently, the effects of dietary Arg on endocrine growth factors, such as GH and IGF-I, in channel catfish [5], Jian carp [32], and gibel carp [6], were shown to accelerate the efficiency of nutrient utilization and growth.

In the present study, NCG significantly improved the growth and feed efficiency of mirror carp (*Cyprinus carpio*). The fish fed the Arg-deficient diets that were supplemented with 0.12% and 0.16% NCG showed significantly higher FWB, WGR or PER values compared with the fish fed the Arg-deficient diet supplemented with 0 NCG. Similarly, Cheng et al. reported that dietary supplementation with 0.2% NCG improved the weight gain and the protein efficiency ratio in Nile tilapia fed Arg normal diets [22]. In mammals, supplementation with NCG is a novel and effective means to enhance piglet growth [3]. Frank found that the oral administration of NCG increased the absolute protein synthesis in the longissimus and gastrocnemius muscles of young pigs by 30 and 21%, respectively [15]. Wu et al. reported that supplementation with NCG to the weaning diet increased the daily weight gain of pigs without affecting their feed intake [16]. This study indicated that supplementation with NCG increased the growth of mirror carp fed an Arg-deficient diet.

4.2. The concentration of Arg and NO and the activity of T-NOS

In the present study, dietary Arg deficiency decreased the concentration of Arg and NO and the activity of T-NOS in the plasma of mirror carp. Arg is the precursor for the synthesis of NO and polyamines [44]. NOS catalyses Arg to produce NO and citrulline. Similarly, Tu et al. found that T-NOS activity was positively correlated with NO [6]. Arg improved the growth and feed efficiency, which could be related to enhanced delivery of amino acids to the muscle as a result of Arg-induced NO synthesis. NO increases muscle blood flow, and this is proposed to be a mechanism responsible for the stimulation of muscle protein synthesis under certain conditions, such as during the infusion of IGF-I [45]. NO is significantly correlated with the SGR of fish, and this may indicate a close relationship between NO and the growth of gibel carp [6].

In the present study, NCG supplementation significantly increased the concentration of Arg, NO and the activity of T-NOS in the plasma. Similarly, Cheng et al. reported that the level of plasma Arg significantly increased in fish fed with 0.4% NCG, which was four times more than the control group in Nile tilapia [22]. In mammals, the supplementation of NCG increases the mRNA expression of the intestinal amino acid transporters *Slc6a19*, *Slc7a9*, and *Slc1a1* and protein abundance of ASCT2, B0AT1, $b^{0,+}$AT, y^{+}LAT1, and EAAC1 in the jejunum of weaned piglets [46]. *Slc6a19* and *Slc1a5*, *Slc7a9* and *Slc7a7*, *Slc1a1*, and *Slc15a1* are identified as the major intestinal transporters for neutral, basic, and acidic amino acids and peptides, respectively [47]. According to previous literature, NCG supplementation increases the concentration of Arg in the plasma, which may be ascribed to the absorptive function by increasing the expression of amino acid transporters in the intestine. On the other hand, the metabolic activation of intestinal citrulline and Arg synthesis may be an attractive approach to augment the Arg provision in reared piglets [3]. The Arg synthetic

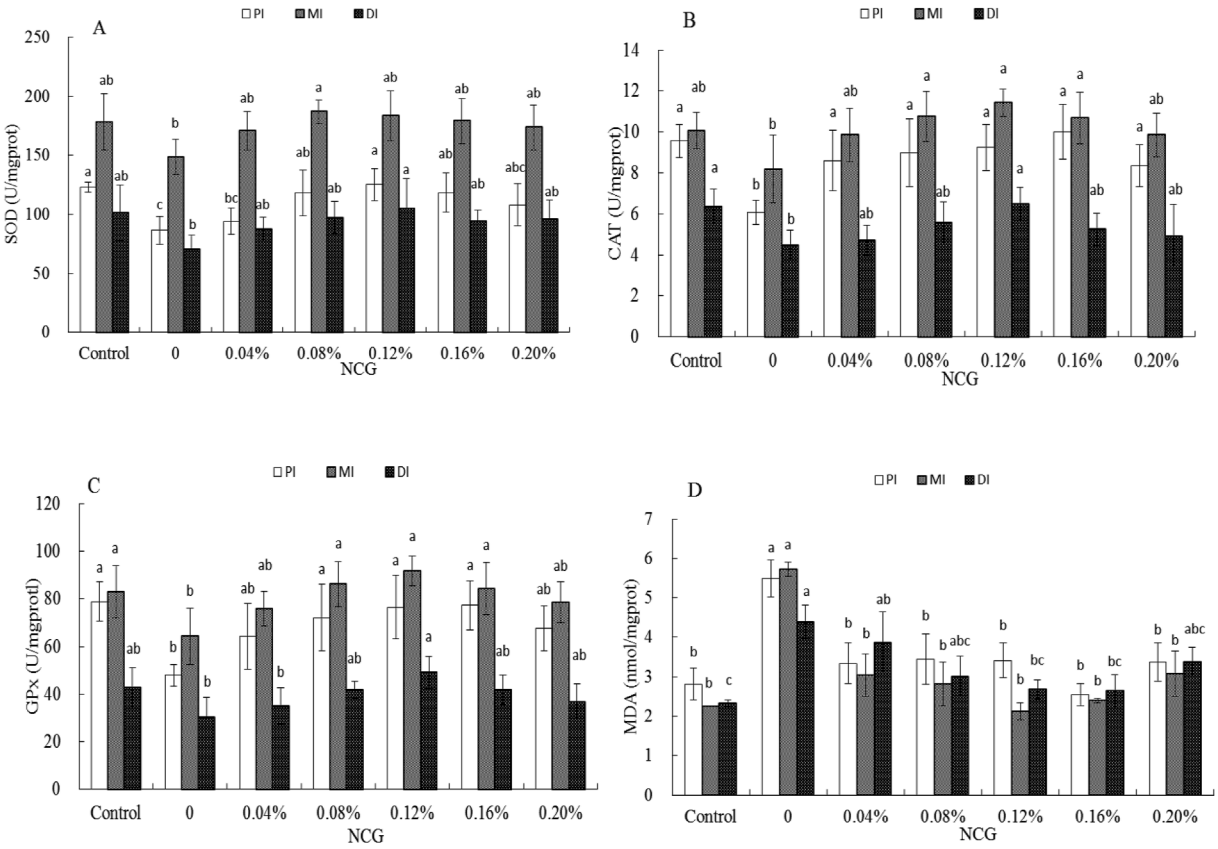

Fig. 2. Effect of NCG on the activities of superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione peroxidase (GPx) (C) and the concentration of malondialdehyde (MDA) (D) in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of mirror carp (*Cyprinus carpio*) under dietary arginine deficiency. The data represent the mean of three replicates. The different small letters indicate significant differences ($P < 0.05$) between the different treatments.

pathway involves the conversion of precursor amino acids, either proline, glutamine, or glutamate, to pyrroline-5-carboxylate (P5C), ornithine, citrulline and finally Arg [48]. N-acetylglutamate (NAG) is an allosteric activator of pyrroline-5-carboxylate synthase (P5CS) and carbamoylphosphate synthase-I (CPS−I) [49], which synthesizes the mitochondrial carbamoyl-phosphate necessary for the conversion of ornithine into citrulline and Arg. However, cells need a high deacylase activity to catabolize NAG [50], thus limiting the use of extracellular NAG to increase mitochondrial NAG concentrations. NCG (an analog of NAG) is a metabolically stable activator of CPS-I. Importantly, Cohen et al. initially discovered the activation of hepatic citrulline synthesis by NCG [51]. Additionally, NCG is not toxic to animals, on the basis of studies with infants [52] and rats [53], and is used to prevent hyperammonemia. Therefore, endogenous Arg synthesis is critical for maintaining Arg homeostasis [54].

4.3. Intestinal antioxidant status

Under normal physiological conditions, animal cells produce reactive oxygen species (ROS). At the same time, the body has designed several antioxidant defense mechanisms. An imbalance between the generation and removal of ROS produces oxidative stress [55]. The antioxidant defenses of fish prevent the uncontrolled generation of ROS by enzymes, such as SOD, CAT and GPx. Assaying antioxidant enzymes offers an indication of the antioxidant status of organisms and serve as a biomarker of oxidative stress. The activities of CAT and SOD are generally used as indicators of the antioxidant defense status of the organism. SOD is a crucial antioxidant defense enzyme that works to ameliorate stress-mediated immunosuppressive responses [56]. SOD catalyses the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and provides an important defense mechanism against superoxide radical toxicity, while CAT and GPx degrade H_2O_2 [57]. Thus, SOD activity reflects the ability to clear ROS and the severity of cell impairment. In the present study, the Arg-deficient diet decreased activities of SOD, CAT and GPx and increased concentration of MDA in the intestine compared with the control diet. Similarly, the dietary Arg deficiency decreases GPx enzyme activity or expression in blunt snout bream [58], Jian carp [24], grass carp [59] and yellow catfish [10]. Furthermore, the SOD activity or expression decreases in Jian carp [24], grass carp [59] and yellow catfish [10] fed an Arg-deficient diet, but it is not affected by the dietary Arg level in blunt snout bream [58]. Arg deficiency decreases the intestinal antioxidant status of fish, which may be ascribed to the upregulation protein kinase C (PKC) and NF-E2-related nuclear factor 2 (Nrf2) expression in fish [24]. Nrf2 is demonstrated to be a critical transcription factor, which regulates the gene expression of antioxidant enzyme genes in fish [60]. In the present study, the Arg-deficient diet supplemented with 0.08% or 0.12% NCG significantly increased the antioxidant activities and decreased the concentration of MDA in the intestine. The reason for this might be that NCG supplementation significantly increased the concentration of Arg and NO in the plasma. Arg stimulates the production of NO within the intestine, and this molecule plays an important role in regulating the antioxidant defense system [26] [61], [62]. Similarly, the concentrations of PC and MDA were lower in the jejunum, duodenum, and ileum of suckling lambs supplemented with 0.1% NCG compared with intrauterine-growth-retarded (IUGR) [27]. MDA is the final product of lipid peroxidation, which leads to cell toxicity and accelerates the damage to cells and tissues [63]. The MDA content reflects the extent of lipid peroxidation [64]. The supplementation NCG significantly increased in the activities of SOD, CAT and GPX and, consequently, decreased in the MDA levels in the intestine.

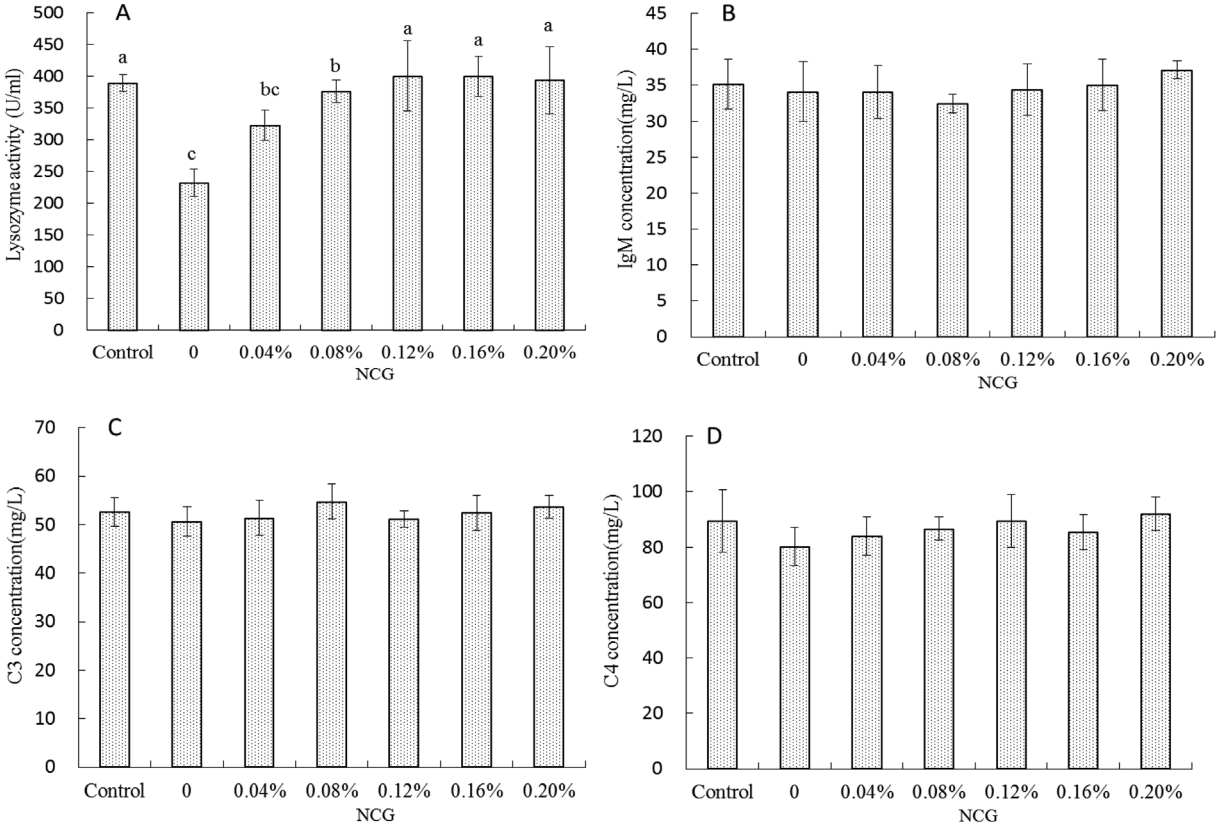

Fig. 3. Effect of NCG on lysozyme activity (A) and the IgM (B), complement 3 (C_3) (C) and complement 4 (C4) (D) concentrations in mirror carp (*Cyprinus carpio*) under dietary arginine deficiency. The data represents the mean of three replicates. The different small letters indicate significant differences ($P < 0.05$) between the different treatments.

4.4. Immune responses

The nonspecific immune system is vitally important for the disease resistance in teleost fish compared with other higher vertebrates [65]. In fish, the humoural components, such as complement, lysozyme and immunoglobulins, play an important role in nonspecific and specific immunity [66]. Lysozyme is a cationic enzyme that attacks the peptidoglycan of bacterial cell walls and acts as nonspecific innate immunity molecule against the incursion of detrimental bacteria [67]. Thus, the alteration in lysozyme activity is usually used as an important index of the innate immunity function [68]. In addition, the complements are major molecules of innate immunity that exert their immune function through the recognition and destruction harmful microbes [69]. IgM is the major component of the specific humoural immune system [70]. Similar to our results, dietary Arg deficiency significantly decreases the lysozyme activity in the plasma compared with the control group [9] [11], [71]. NCG was proven to increase the endogenous synthesis of Arg, thus, dietary NCG supplementation may help alleviate deficiencies in Arg of mirror carp fed an Arg-deficient diet. In the present study, dietary NCG supplementation significantly increased the plasma lysozyme activity in mirror carp compared to the Arg-deficient group. One reasonable explanation for these results is probably was that NCG supplementation significantly increases the concentration of Arg, which is identified as a potential modulator of both the innate and adaptive immune systems in vertebrates [72]. In addition, dietary Arg supplementation increases the plasma lysozyme activity in red drum [73], hybrid striped bass [74] and turbot [75], [76]. However, the IgM, C3, and C4 concentrations of mirror carp were not significantly influenced by the different levels of dietary Arg or NCG. This finding was inconsistent with previous research, reporting that Arg supplementation led to increased IgM, C3, and C4 concentrations [71] [77], the difference may be due to the above experiment was conducted by injection of *Aeromonas* hydrophila or *Edwardsiella ictaluri*.

In fish, the immune status is closely associated with inflammation, which is initiated and regulated by inflammatory cytokines [78]. TNF-α and IL-1β are two well-studied cytokines in fish [79], [80]. TNF-α takes part in the inflammatory response, cell proliferation and the stimulation of the immune system [81]. IL-1β is a typical pro-inflammatory cytokine in the fish immune response, and it induces macrophages and lymphocytes to release cytokines when pathogens invade the organism [82]. IL-8 is an important pro-inflammatory cytokine that recruits and activates macrophages and neutrophils to remove cell debris and invades microorganisms and facilitates the regrowth of injured tissues [83]. An *in vitro* study showed that NO production in channel catfish macrophages is significantly influenced by Arg [84]. In the present study, Arg deficiency significantly increased the expression of TNF-α, IL-1β and IL-8 in the intestine compared with the control group. Analogous to this study, dietary Arg deficiency increased the expression or content of IL-8 and TNF-α in blunt snout bream [58] and increased the expression of IL-1β in jian carp [24], [71]. In carp, dietary Arg decreases the expression of pro-inflammatory cytokine genes (IL-1β and TNF-α) in the LPS-induced inflamed intestine [25]. The results indicate that dietary Arg deficiency promotes the inflammatory response partly by upregulating the mRNA levels of pro-inflammatory factors in fish. In the present study, the result showed that the levels of inflammatory-related cytokines were down-regulated, including TNF-α, IL-8 and IL-1β, compared to the fish fed the Arg-deficient diet, indicating the protective effects of the NCG against the dietary Arg deficiency-induced inflammatory response in the intestine of common carp. In fish, macrophage NO production plays an important role in the cellular defense mechanism and is demonstrated in stimulated macrophages in several fish species [61], [85]. One reasonable explanation for these results

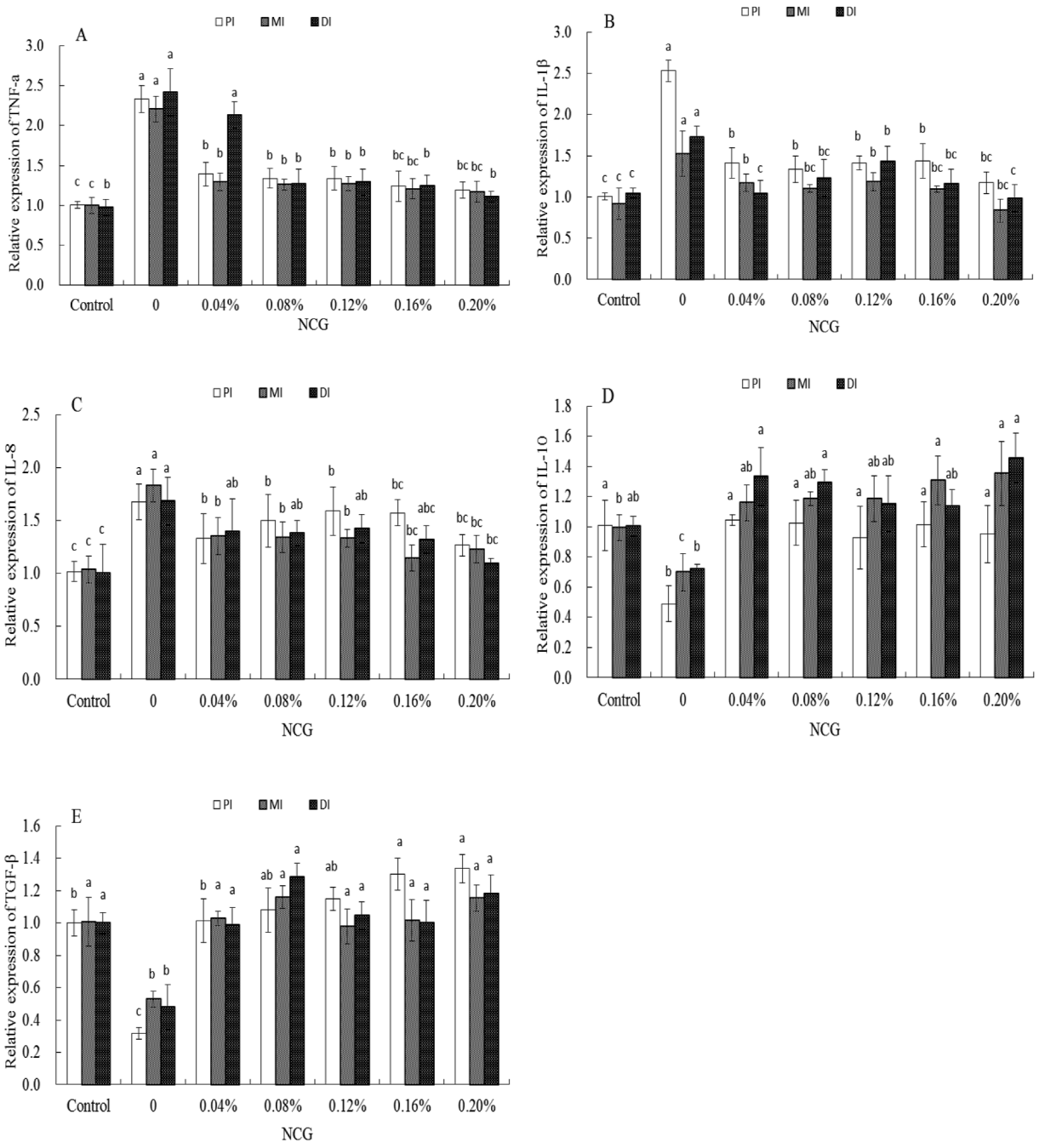

Fig. 4. Effect of NCG on the relative mRNA expression of tumor necrosis factor-α (TNF-α) (A), interleukin1β (IL-1β) (B) and interleukin 8 (IL-8) (C), interleukin 10 (IL-10) (D) and transforming growth factor β (TGF-β2) (E) in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) in mirror carp (*Cyprinus carpio*) under dietary arginine deficiency. The data represent the mean of three replicates. The different small letters indicate significant differences ($P < 0.05$) between the different treatments.

probably is that the dietary supplementation of NCG enhances the release of NO from macrophages [86], which decreases the release of pro-inflammatory cytokines, such as TNF-α [87] and IL-1β [88]. Furthermore, common carp fed the NCG diet show down-regulated levels of the anti-inflammatory factors IL-10 and TGF-β2. IL-10 and TGF-β are important anti-inflammatory cytokines, which counteract the production of pro-inflammatory cytokines and limit the inflammatory response [89]. The prime function of IL-10 is to neutralize pro-inflammatory cytokines (e.g., IL-1β and TNF-α), thereby preventing tissue injury [90]. Similarly, the ileum SIgA, IL-10 levels, and CD4% in NCG-treated piglets are higher than in no-NCG-treated piglets [17]. These results indicate that an Arg-deficient diet supplemented with NCG improves the nonspecific immunity by increasing the activity of lysozyme, the relative mRNA expression of IL-10 and TGF-β2 and decreasing the relative mRNA expression of TNF-α, IL-1β and IL-8 of fish.

In conclusion, dietary Arg deficiency induced intestinal inflammation by regulating the mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) and anti-inflammatory cytokines (TGF-β2 and IL-10) in mirror carp and decreased the growth performance. Additionally, dietary 0.12% or 0.16% NCG improved the growth performance, feed utilization, intestinal antioxidant capacity and immune response in the fish fed the Arg-deficient diet.

The data are the mean values ± SEM of three replicates. The means in the same row, with different superscripts, are significantly

($P < 0.05$) different.

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