ORIGINAL ARTICLE

Aquaculture Research WILEY

Effects of dietary arginine supplementation on cytokine- and antioxidant-related gene expressions in common carp (*Cyprinus carpio*) fingerling during ammonia toxicity

Morteza Yousefi[1] | Behrooz Abtahi[2] | Hossein Adineh[3] | Seyed Hossein Hoseinifar[4] | Ali Taheri Mirghaed[5] | Marina Paolucci[6] | Hien Van Doan[7,8]

[1]Department of Veterinary Medicine, Peoples' Friendship University of Russia, RUDN University, Moscow, Russia

[2]Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran

[3]Department of Fisheries, Faculty of Agriculture and Natural Resources, Gonbad Kavous University, Gonbad Kavous, Iran

[4]Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

[5]Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[6]Department of Sciences and Technologies, University of Sannio, Benevento, Italy

[7]Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand

[8]Science and Technology Research Institute, Chiang Mai University, Chiang Mai, Thailand

Correspondence
Hien Van Doan, Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand.
Email: hien.d@cmu.ac.th

Funding information
Chiang Mai University; RUDN University

Abstract

This study investigated the effects of dietary arginine supplementation on plasma ammonia and urea levels, and immune- and antioxidant-related gene expressions of common carp (*Cyprinus carpio*), exposed to ambient ammonia. Fish (10.5 ± 0.74 g) were fed diets containing arginine (0: control diet, 0.25: 0.25Arg and 0.5%: 0.5Arg) for 14 days and then subjected to ammonia exposure for three hours. The results showed that arginine significantly decreased plasma ammonia level, whereas increased the plasma urea level. Arginine supplementation significantly up-regulated head kidney *il1b*, *il10*, *tnfa* and liver *sod*, *cat*, *gpx* and *gst* gene expressions, whereas significantly down-regulated *hsp70* gene expression in liver. Ammonia exposure led to a significant increase in plasma ammonia and urea levels. There were elevations in head kidney *il1b*, and liver *sod*, *cat*, *gpx*, *gst* and *hsp70* gene expression in fish after challenged with ammonia. The interaction effects of arginine supplementation and ammonia exposure on head kidney *il10*, and liver *gst* and *hsp70* gene expressions were observed, as arginine prevented ammonia-induced down-regulation in *il10* expression, mitigated ammonia-induced up-regulation in *hsp70* expression and intensified up-regulation in *gst* expression. In conclusion, it is suggested that two-week supplementation of arginine (0.5% of diet) is useful to mitigate the adverse effects of ambient ammonia when in the farm, common carp is at risk of ammonia toxicity.

KEYWORDS
arginine, common carp, cytokine, health, oxidative stress

1 | INTRODUCTION

Common carp (*Cyprinus carpio*) is one of the most popular freshwater fish species with high demands around the world. Contribution of common carp in the total production of freshwater fish is considerable (about 71.9%); thus, its production in intensive farming systems has been of interest to fish farmers to meet the demands (Abdel-Tawwab & Monier, 2018). However, intensive culture may lead to accumulation of organic residues and toxic inorganic ions. Unionized ammonia (NH_3) is one of the most important toxins

known in farmed fish species (Randall & Tsui, 2002). In addition to being the main part of the nitrogenous wastes of fish, it can be produced by the accumulation of organic matters and decomposing of un-eaten feed in water environment (Adineh et al., 2019; Liu et al., 2008). Numerous studies on fish have demonstrated that high levels of ambient ammonia disrupt internal ammonia excretion, leading to hyperammonemia (McKenzie et al., 2009; Yousefi et al., 2020). Hyperammonemia may suppress immune function (Hoseini et al., 2019a; Taheri Mirghaed et al., 2019), antioxidant capacity (Taheri Mirghaed et al., 2018; Yousefi et al., 2020) and inflammation (Cheng et al., 2015; Zhang et al., 2018). Ammonia toxicity in fish may be counteracted via two methods, first, by augmenting fish antioxidant and immune responses to minimize the toxic effects of ammonia, and second, by increasing the detoxification rate in the fish body (Hoseini et al., 2019a). Fish antioxidant and immune systems are crucial to maintain fish health, and there are several reports about the use of dietary additives to augment these systems. Among the feed additives, amino acids are important immunostimulants and antioxidants (El-Sayed, 2014; Hoseini et al., 2019c, 2020).

Arginine is a necessary amino acid in fish with wide range of functions including growth promotion, immunostimulation and antioxidant effects (Coutinho et al., 2016; Wang et al., 2015; Zhou et al., 2015). Besides, arginine was found to suppress inflammation when the fish face inflammatory conditions, such as lipopolysaccharide induction (Holen et al., 2014; Jiang et al., 2015). Therefore, dietary arginine administration may suppress immunosuppression, inflammation and oxidative stress caused by ammonia exposure in fish. Moreover, arginine may accelerate ammonia detoxification in fish, via the urea cycle (Hoseini, Khan, et al., 2020). Some fish species, including common carp (Diricx et al., 2013), are able to convert ammonia to urea via sequential enzymatic stages; urea is less toxic to fish, compared with ammonia. For this, ammonia reacts with carbamoyl phosphate and ornithine, resulting in citrulline formation; the compound is converted to arginine and urea in subsequent steps (Hoseini, Khan, et al., 2020). Arginine availability is necessary for ornithine production, which pushes the urea cycle forward for more ammonia detoxification. Therefore, arginine administration may be useful for ammonia detoxification in fish. According to above, dietary arginine administration might be a suitable dual-effect strategy to mitigate ammonia toxicity in fish, which needs to be studied. Therefore, the current research investigated the potential effects of different levels of dietary arginine on ureagenesis and its association to immune and antioxidant responses at transcriptomic levels in common carp during ammonia toxicity.

2 | MATERIALS AND METHODS

2.1 | Diet preparation

For diet preparation, feedstuffs (Table 1) were sieved to remove large particles. Then, based on the fish requirement, dietary formulation was prepared. The feedstuffs were mixed well for 30 min, and then,

TABLE 1 Ingredients, chemical composition and amino acid profile of the diets supplemented with 0, 0.25 and 0.5% arginine

Ingredients (g/kg)	Dietary arginine (%)		
	0	0.25	0.5
Soya bean meal[a]	150	150	150
Fish meal[b]	250	250	250
Poultry meal[c]	250	250	250
Wheat meal	340	337.5	335
Lysine[d]	2	2	2
Methionine[e]	2	2	2
Mineral mix[f]	3	3	3
Vitamin mix[g]	3	3	3
Arginine	0	2.5	5
Proximate composition			
Moisture (%)	9.52	9.68	9.75
Crude protein (%)	40.1	39.9	40.3
Crude lipid (%)	9.88	9.92	10.0
Crude ash (%)	10.5	10.0	10.8
Amino acid composition (% of dietary protein)			
ARG	3.42	4.00	4.63
ASP	4.32	4.25	4.56
GLU	15.3	16.3	17.2
SER	4.60	4.39	4.75
HIS	1.88	1.77	1.89
GLY	5.03	5.45	5.65
THR	2.95	2.88	2.78
ALA	3.65	3.28	3.65
TYR	3.21	3.45	3.65
MET	2.85	2.75	2.81
VAL	4.17	4.12	4.32
PHE	4.62	4.55	4.42
ILE	2.40	2.32	2.45
LEU	5.82	6.00	5.88
LYS	4.95	4.85	4.96

[a]Gorgan Soya (Gorgan, Iran); crude protein: 44%.
[b]Sardine fish meal; crude protein: 59%.
[c]Peyar Slaughter (Gorgan, Iran); crude protein: 49%.
[d]Faravar Lysine Pars.
[e]Mad Tiour.
[f]The premix provided following amounts per kg of feed: A: 1000 IU; D_3: 5000 IU; E: 20 mg; B_5: 100 mg; B_2: 20 mg; B_6: 20 mg; B_1: 20 mg; H: 1 mg; B_9: 6 mg; B_{12}: 1 mg; B_4: 600 mg; C: 50 mg.
[g]The premix provided following amounts per kg of diet: Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; Se: 0.3 mg; I: 1.5 mg; Mn: 10 mg).

water (200 ml/kg) was added to form dough. The dough was pelleted by laboratory pellet machine (La Monferrina P3). Crystalline L-arginine (Sigma-Aldrich) was added to other feedstuffs at the rates of 0 (Arg0), 0.25 (Arg0.25) and 0.5% (Arg0.5) (Hoseini et al., 2019). The required arginine amount was mixed with the vitamin and mineral premix

before adding to the feedstuff mixture. The diets were dried against fan blow (22°C) and kept at −20°C for further use. The proximate composition of the diets was determined based on AOAC (2005).

2.2 | Fish and experimental design

Common carp advanced fingerlings (90 individuals) with a mean weight of 10.5 ± 0.74 g were supplied from a local farm. Fish were fed on basal diet and acclimated to laboratory experimental condition for 10 days. Then, fish were introduced into nine 60-L tanks (10 fish per tank; water flow rate: 0.3 L/min. kg biomass) and fed their respective diets twice a day at a rate of 2.5% of body weight/day for 14 days. Water quality parameters including temperature (23.2–23.9°C), pH (7.55–7.63), un-ionized ammonia (0.0005–0.001 mg/L) and alkalinity (177–183 mg/L) were monitored daily. At the end of feeding trial, fish of each treatment were challenged with 0.7 mg/L concentrations of water unionized ammonia (ammonium chloride) for 3 h. Total ammonia, pH and temperature values were used to determine water unionized ammonia as described by Emerson et al. (1975). All parts of the study were conducted under a protocol approved by the committee of ethics of the faculty of sciences of the University of Tehran (357; 8 November 2000).

2.3 | Tissue sampling and analysis

Before (CTL) and after challenge with ammonia (AMM), two fish were sampled from each tank and immediately anaesthetized (100 mg/L eugenol solution). Using heparinized syringes, blood samples were taken from the caudal vein and poured into plastic tubes. To obtain plasma, the samples were centrifuged for 10 min (1200 g) and resultant kept at −70°C for the future analysis. After blood sampling, fish were killed by spinal cord dissecting. Liver and kidney were removed, snap-frozen in liquid nitrogen and stored at −70°C until analysis.

Plasma levels of ammonia (Greiner Diagnostic GmbH) and urea (Pars Azmun) were determined using available commercial kits by an auto-analyzer (Hitachi 717).

Kidney and liver tissues samples (100 mg) were utilized to isolate the total RNA using a commercial kit (Gene All Kit, Gene All Biotechnology). Then, the purity and quantity of obtained RNA were determined spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific) at 260/280 nm. The synthesize the first-strand Complementary DNA (cDNA) was performed by reverse transcription using a commercial kit (transcription Kit, GeneAll Biotechnology) according to the manufacturer's protocol. Designing the primer sets for quantification of mRNA levels of selected genes [catalase (*cat*), superoxide dismutase (*sod*), glutathione peroxidase (*gpx*), glutathione S-transferase (*gst*), heat shock protein (*hsp70*), interleukin-1 beta (*il1b*), interleukin-10 (*il10*) and tumour necrosis factors-alpha (*tnfa*)], was performed using the sequences for common carp found in GenBank (Table 2). The quantitative real-time PCR assay was performed to determine the relative expression of the selected genes using the SYBR green method. The expression levels of selected genes were normalized using *beta-actin* gene as the housekeeping gene. The relative mRNA levels were analysed based on the method described by Livak and Schmittgen (2001).

2.4 | Statistical analysis

All data are expressed as mean ± standard error (SE). Kolmogorov–Smirnov test was used to assess the normality of data distributions.

TABLE 2 Primer sequences and accession number of gene selected for real-time PCR

Gene	Sequences	Amplicon	Melting temperature	Accession no.	Efficiency %
beta-actin	F: CCTGTATGCCAACACCGTGCTG R: CTTCATGGTGGAGGGAGCAAGG	103	61	XM_019089433.1	93
cat	F: AGACGACACCCATCGCTGTTCG R: AAGGTCCCAGTTGCCCTCATCG	122	61	GQ376154.1	98
sod	F: TGGCGAAGAAGGCTGTTTGT R: TTCACTGGAGACCCGTCACT	91	61	XM_019111527.1	98
gpx	F: CTCAACAGGAGAATGCCAAGAATG R: CCTTGAGGAACACGAACAGAGG	147	61	XM_019093635.1	97
gst	F: CGTGTGTCAGAGTCAGCGT R: GGAGCCCCAGTACAACATC	138	61	XM_019117245.1	99
hsp70	F: TGTGAGCGAGCCAAGAGAACCC R: AAGCGAGCTCTGGTGATGGACG	107	61	XM_019074376.1	96
il-1b	F: ACCAGCTGGATTTGTCAGAAG R: ACATACTGAATTGAACTTTG	465	61	XM_019080073.1	98
il-10	F: CGCCAGCATAAAGAACTCGT R: TGCCAAATACTGCTCGATGT	103	61	JX524550.1	97
tnfa	F: GGTGATGGTGTCGAGGAGGAA R: TGGAAAGACACCTGGCTGTA	348	61	XM_019088899.1.1	99

Relationships between dietary arginine with plasma ammonia and urea levels were determined by regression. Effect of dietary arginine inclusion at different levels and ammonia exposure was tested using two-way ANOVA. When there was an interaction effect of arginine levels × ammonia exposure, one-way ANOVA was used for the data analysis. Duncan test was performed to determine a significant difference among the treatments. All data were analysed using SPSS 16.0 (SPSS).

3 | RESULTS

The fish exhibited elevated plasma ammonia (Figure 1; $p < 0.001$) and urea (Figure 2; $p < 0.001$) levels, when exposed to ambient ammonia. Dietary arginine supplementation significantly ($p < 0.001$) decreased plasma ammonia level, as Arg0.5 treatment presented significantly lower plasma ammonia level compared with the other treatments (Figure 1). Increase in dietary arginine level significantly increased plasma urea concentration, as the lowest and highest levels were observed in the Arg0 and Arg0.5 treatments (Figure 2). There were no interaction effects of ammonia exposure and dietary arginine supplementation on plasma ammonia and urea levels; however, there were significant correlations between dietary arginine with plasma ammonia ($R^2 = 0.93$–0.99) or urea ($R^2 = 0.99$) levels both before and after ammonia exposure (Figures 1 and 2).
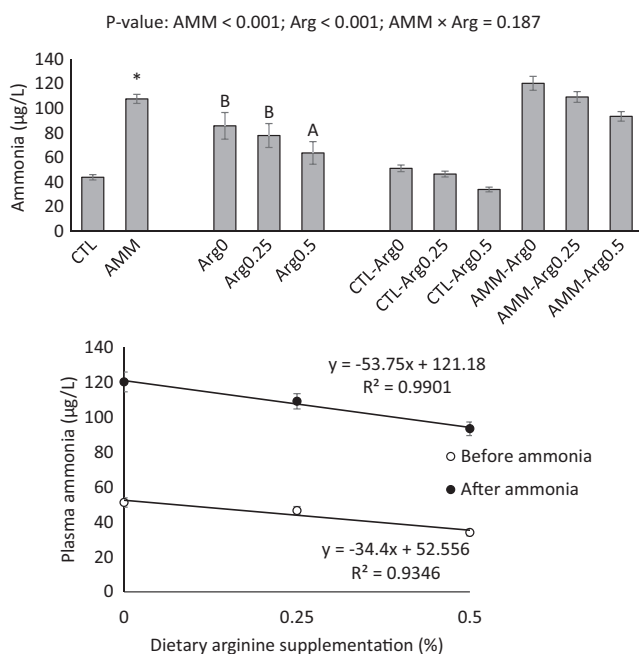
FIGURE 1 The upper figure: Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on ammonia levels (mean ± SE) in the blood plasma of common carp. The asterisk shows significant effects of ammonia exposure ($n = 18$). Uppercase letters show significant effects of dietary Arg levels ($n = 12$). Lowercase letters show significant interaction effects of dietary Arg levels and ammonia exposure ($n = 6$). The lower figure: Relationships between dietary arginine with plasma ammonia levels in the fish before and after ammonia exposure ($n = 36$)

Ammonia exposure induced no significant change in head kidney *tnfa* gene expression ($p = 0.293$), whereas dietary arginine significantly ($p < 0.001$) changed the gene expression, as the Arg0.5 treatment presented significantly higher gene expression compared with the other treatments (Figure 3). Ammonia exposure and dietary arginine induced significant interaction effects on head kidney *il1b* gene expression ($p < 0.001$). Before ammonia exposure, the Arg0.25 and Arg0.5 exhibited significant up-regulations in the gene expression and the highest expression was observed in the Arg0.5 treatment. Ammonia exposure significantly up-regulated the gene expression in the Arg0 and Arg0.25, but not Arg0.5 treatments. Meanwhile, arginine supplementation induced no significant change in the gene expression, after ammonia exposure (Figure 3). Ammonia exposure and dietary arginine induced significant interaction effects on head kidney *il10* gene expression ($p < 0.001$). There was no significant difference in the gene expression, before ammonia exposure; nevertheless, ammonia exposure led to down-regulation in the gene expression in the Arg0 treatment, but not the other treatments (Figure 3).

Ammonia exposure and dietary arginine supplementation significantly ($p < 0.001$) affected liver *sod* gene expression. The fish presented higher gene expression, after ammonia exposure; moreover, increase in dietary arginine levels significantly up-regulated *sod* gene expression (Figure 4). Ammonia exposure significantly up-regulated *cat* gene expression ($p < 0.001$). Dietary arginine supplementation presented significant effects ($p = 0.003$) on liver *cat* expression and Arg0.5 had significantly higher gene expression compared with the other treatments (Figure 4).

Ammonia exposure and dietary arginine had interaction effects on liver *gpx* ($p = 0.015$) and *gst* ($p < 0.001$) gene expressions (Figures 5 and 6). Before ammonia exposure, *gpx* gene expression significantly increased along with dietary arginine elevation. After ammonia exposure, the Arg0.25 and Arg0.5 exhibited higher gene expression compared with the Arg0 treatment. Meanwhile, all the ammonia-exposed fish exhibited significant up-regulations in *gpx* gene expression. There were no significant differences in *gst* gene expression among the treatments before ammonia exposure. However, the gene expression significantly increased along with dietary arginine elevation, after ammonia exposure. Here again, all the ammonia-exposed fish exhibited significant up-regulations in *gst* gene expression (Figures 6).

Ammonia exposure and dietary arginine had an interaction effect on liver *hsp70* ($p < 0.001$) gene expression (Figure 5). There were no significant differences in *hsp70* gene expression among the treatments before ammonia exposure. Ammonia exposure significantly up-regulated the gene expression in all treatments, with the lowest up-regulation in Arg0.5 and Arg0.25 treatments.

4 | DISCUSSION

Elevated level of ambient ammonia results in hyperammonemia in fish, which in turn, leads to decline in fish health, characterized by

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg = 0.101

P-value: AMM = 0.293; Arg < 0.001; AMM × Arg = 0.093

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg < 0.001

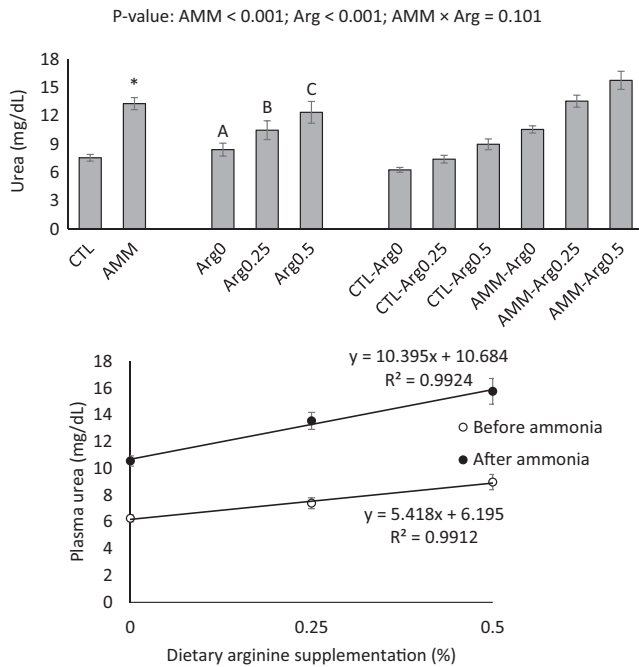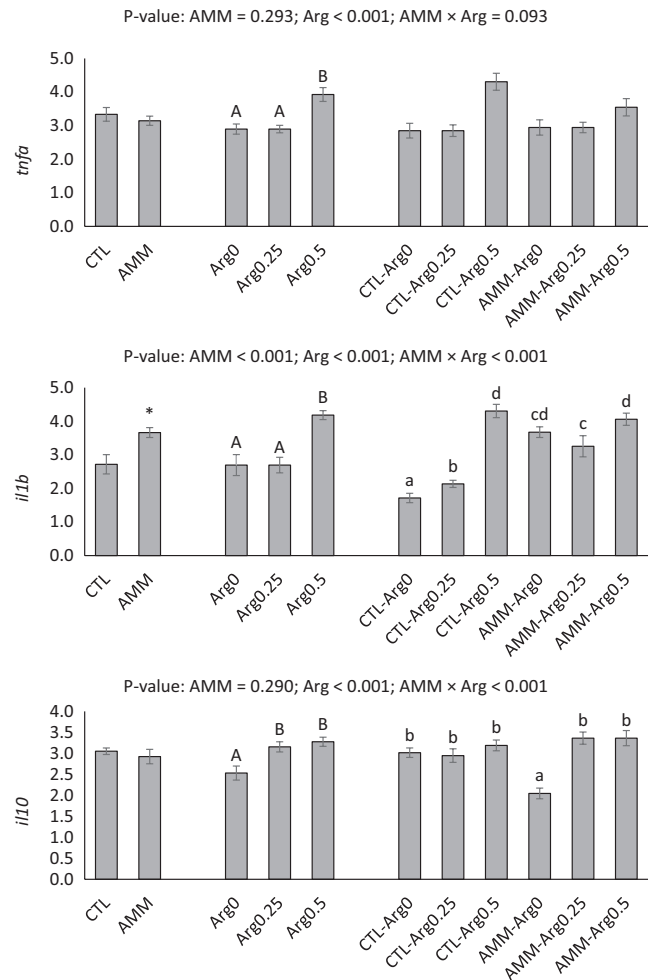P-value: AMM = 0.290; Arg < 0.001; AMM × Arg < 0.001

Fig. 2

FIGURE 2 The upper figure: Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on urea levels (mean ± SE) in the blood plasma of common carp. The asterisk shows significant effects of ammonia exposure ($n = 18$). Uppercase letters show significant effects of dietary Arg levels ($n = 12$). Lowercase letters show significant interaction effects of Arg levels and ammonia exposure ($n = 6$). The lower figure: Relationships between dietary arginine with plasma ammonia levels in the fish before and after ammonia exposure ($n = 36$)

FIGURE 3 Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on *tnfa*, *il1b* and *il10* gene expression in common carp kidney. The asterisk shows significant effects of ammonia exposure ($n = 18$). Uppercase letters show significant effects of dietary Arg levels ($n = 12$). Lowercase letters show significant interaction effects of Arg levels and ammonia exposure ($n = 6$)

immunosuppression and oxidative stress (Rajabiesterabadi et al., 2020). However, some fish species are able to convert ammonia to urea in the enzymatic urea cycle. The present results showed that common carp did this, as elevation in plasma urea level was observed after ammonia exposure. The results are in line with the previous studies on the same species (Hoseini, Vatnikov, et al., 2019; Taheri Mirghaed et al., 2018, 2019). Arginine was found to stimulate urea cycle, leading to increased ureogenesis rate in different fish species (Hoseini, Khan, et al., 2020), including common carp (Hoseini, Vatnikov, et al., 2019) that supports the present results. Thus, the present study demonstrated that dietary arginine supplementation is a useful method to lower internal ammonia load in common carp during ammonia exposure, which provides health benefits for the fish (see below).

Cytokines are important molecules in inflammatory responses that modulate immune responses. Pro-inflammatory cytokines such as *tnfa* and *il1b* are involve in cell differentiation and migration, apoptotic cell death, among others (Secombes et al., 2001). However, up-regulation in pro-inflammatory cytokine genes might be an indicator of inflammation and it has been demonstrated that toxicants may induce inflammation in fish (Fazelan et al., 2020; Rajabiesterabadi et al., 2020) and this is the case for ammonia toxicity, as well (Cheng

et al., 2015; Zhang et al., 2018). Thus, such data should be interpreted in accordance to experimental contexts. The present results are in line with Chen et al. (2015) and Hoseini, Vatnikov, et al. (2019) that found up-regulations in *tnfa* and *il1b* in common carp head kidney following dietary arginine administration. Such effects of arginine on pro-inflammatory cytokines, under normal conditions, could be interpreted as empowerment of immune responses that prepare the fish to face pathogens. Supporting this hypothesis, Chen et al. (2015) found up-regulation in pro-inflammatory cytokine along with higher survival against *Aeromonas hydrophila* in common carp. On the other hand, ammonia exposure induced up-regulation in *il1b* gene expression in the carp that is a sign of inflammation; particularly when the down-regulation in *il10* gene expression is taken into account. Such results are in line with previous studies that showed ammonia exposure-induced inflammation in fish (Chen et al., 2015; Zhang et al., 2018). Besides, arginine was found to protect the common carp against inflammation induced by ammonia exposure,

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg = 0.470

P-value: AMM < 0.001; Arg = 0.003; AMM × Arg = 0.059
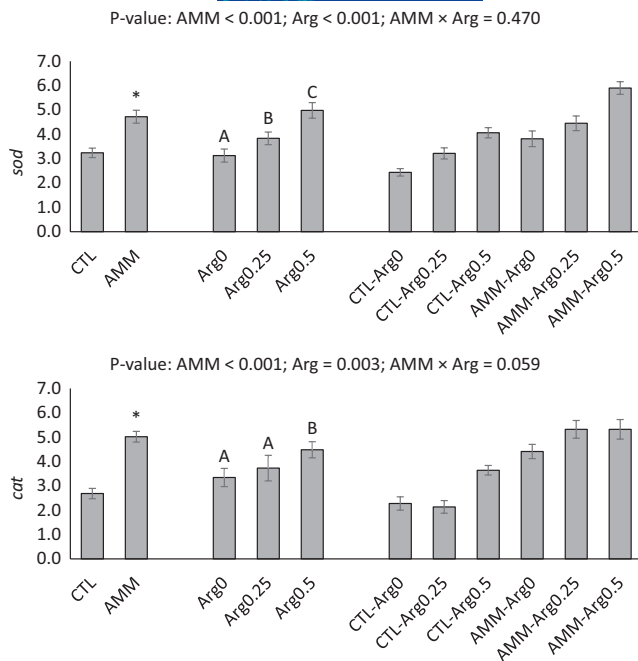
FIGURE 4 Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on *sod* and *cat* gene expression in common carp liver. The asterisk shows significant effects of ammonia exposure (*n* = 18). Uppercase letters show significant effects of dietary Arg levels (*n* = 12). Lowercase letters show significant interaction effects of Arg levels and ammonia exposure (*n* = 6)

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg = 0.015

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg < 0.001
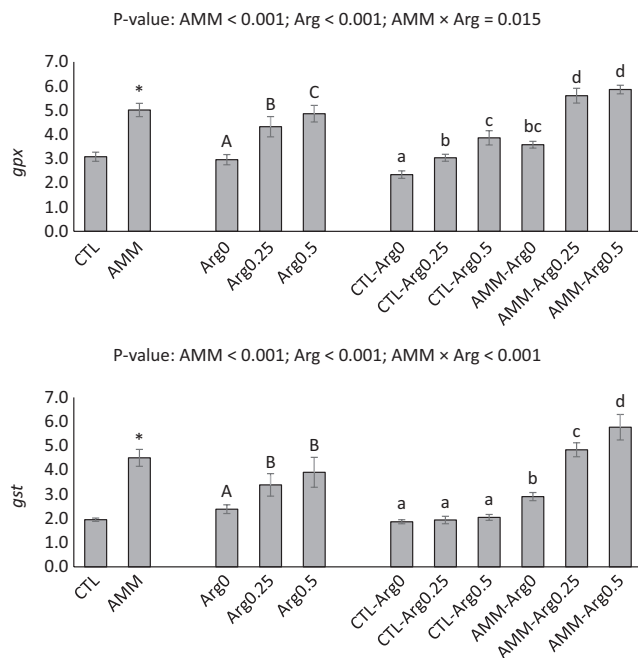
FIGURE 5 Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on *gpx* and *gst* gene expression in common carp liver. The asterisk shows significant effects of ammonia exposure (*n* = 18). Uppercase letters show significant effects of dietary Arg levels (*n* = 12). Lowercase letters show significant interaction effects of Arg levels and ammonia exposure (*n* = 6)

P-value: AMM < 0.001; Arg < 0.001; AMM × Arg < 0.001
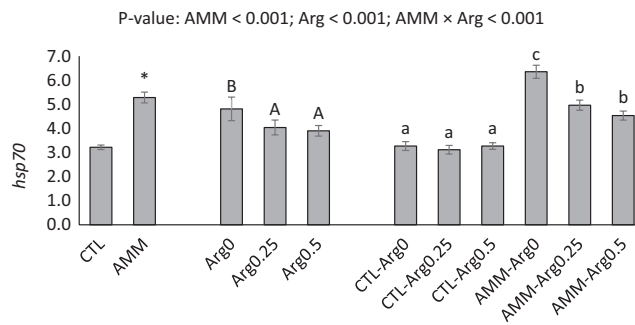
FIGURE 6 Effects of dietary Arg and ammonia exposure (CTL: before ammonia exposure; AMM: after ammonia exposure) on *hsp70* gene expression in common carp liver. The asterisk shows significant effects of ammonia exposure (*n* = 18). Uppercase letters show significant effects of dietary Arg levels (*n* = 12). Lowercase letters show significant interaction effects of Arg levels and ammonia exposure (*n* = 6)

as it restored *il10* gene expression after the ammonia challenge. Supporting these results, Holen et al. (2014) and Jiang et al. (2015) found arginine had no effects on *il10* gene expression under normal condition, but mitigated down-regulation in the gene expression under lipopolysaccharide induction. Overall, the present results showed that arginine administration may improve immune strength in carp under normal condition and mitigate inflammation after ammonia exposure.

There are several studies on fish antioxidant system responses to dietary arginine supplementation [reviewed by Hoseini, Khan, et al. (2020)]. Arginine deficiency induces oxidative stress and decline in antioxidant enzymes' activity in common carp (Wang et al., 2019). On the other hand, it was reported that arginine is necessary for high antioxidant enzymes' activity and/or gene expression in grass carp (Wang et al., 2015). Such changes in antioxidant enzymes' gene expression are the downstream effects of dietary arginine on NF-E2-related nuclear factor 2 and Kelch-like ECH-associated protein 1 gene expression (Wang et al., 2015).

Xenobiotic exposure leads to formation of reactive oxygen species, which causes oxidative stress and inflammation (Ghelichpour et al., 2019). Heat shock proteins are important in protecting living cells against stressful conditions such as oxidative stress (Hoseini et al., 2020; Ikwegbue et al., 2018). The present results are in line with Cheng et al. (2015), which found up-regulation in antioxidant-related and heat shock protein gene expressions in *Takifugu obscurus* during ammonia exposure. In the present study, arginine supplementation mitigated oxidative stress in common carp after ammonia exposure, as it stimulated antioxidant-related gene expressions and down-regulated *hsp70* gene expression after ammonia exposure. *sod*, *cat* and *gpx* are necessary for pro-oxidant neutralization, and *gst* has an important role in xenobiotic detoxification (Khalili et al., 2020). Supporting this hypothesis, the arginine-treated fish exhibited significantly lower *hsp70* gene expression, an indicator of oxidative stress (Ghelichpour et al., 2020). Therefore, it is concluded that arginine administration improved antioxidant enzymes before ammonia exposure and augmented

antioxidant power after the exposure, leading to superior performance against ammonia toxicity.

5 | CONCLUSION

Dietary arginine administration is useful to trigger immune and antioxidant responses at transcriptional levels. Moreover, the amino acid can activate the urea cycle during ammonia exposure, which lowers internal ammonia load and its harmful effects. Therefore, arginine is a dual-acting amino acid in fish during ammonia toxicity, as it increases ammonia detoxification and combats its harmful effects through activation of the antioxidant and immune system. Based on the present results, dietary arginine level of 0.5% is recommended for feed formulation, when common carp is at risk of ammonia toxicity.

CONFLICT OF INTEREST
There is no conflict of interest about this article.

AUTHOR CONTRIBUTIONS
Morteza Yousefi and Behrooz Abtahi: Study design, Conceptualization. Hossein Adineh and Seyed Hossein Hoseinifar: Lab analysis. Ali Taheri Mirghaed: Data analysis, Marina Paolucci: Writing - Original Draft. Hien Van Doan: Funding acquisition.

DATA AVAILABILITY STATEMENT
Research data are not shared.

ORCID
Morteza Yousefi https://orcid.org/0000-0001-5352-8106
Seyed Hossein Hoseinifar http://orcid.org/0000-0002-0210-9013
Ali Taheri Mirghaed https://orcid.org/0000-0001-5568-1390
Marina Paolucci https://orcid.org/0000-0002-1784-3843

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