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Abstract

A 12-week feeding trial was conducted to investigate the effects of dietary arginine (Arg), glutamine (Gln), and their interactions on growth performance, nonspecific immunity, disease resistance, and Arg catabolism in juvenile turbot (*Scophthalmus maximus* L.). The basal diet was formulated with white fish meal, soybean meal, and corn gluten meal to contain 50% crude protein and 12% crude lipid. Nine isoproteic and isolipidic experimental diets were formulated by supplementing the basal diet with three levels of L-Arg and L-Gln (0.0%, 1.0%, and 2.0% each). Triplicate groups of 35 fish were fed a fixed ration twice daily in an indoor recirculating seawater system. After the feeding trial, fish were challenged by *Edwardsiella tarda* and survival rate (SR) was recorded for 7 days. The results showed that dietary Arg and Gln levels did not significantly affect growth performance of juvenile turbot ($P>0.05$). Respiratory burst activity in head-kidney macrophages increased significantly as dietary Arg or Gln increased ($P<0.05$), but no significant interactions was observed ($P>0.05$). Significant interactions between dietary Arg and Gln were observed on lysozyme and glutathione peroxidase activity in serum ($P<0.05$). There was also a significant interactive effect of dietary Arg and Gln on inducible nitric oxide synthase activity in serum and liver, and arginase I gene expression in liver ($P<0.05$). Fish fed diets with the highest Arg and Gln showed significantly higher levels of arginase I gene expression in liver ($P<0.05$). After the *E. tarda* challenge, SR of juvenile turbot were significantly improved as dietary Arg increased ($P<0.05$), but no significant effect of dietary Gln and their interactions was observed ($P>0.05$). It can be concluded that inclusion of Arg and Gln in the diet did not have a positive effect on growth performance of juvenile turbot, but significantly improved nonspecific immune responses. Dietary Arg and Gln levels also had significantly interactive effects on Arg

catabolism. Disease resistance was significantly enhanced with the increasing of dietary Arg.

*Keywords***:** arginine, glutamine, juvenile turbot, nonspecific immunity, disease resistance, arginine catabolism

1. Introduction

Global aquaculture is undergoing rapid development accompanied by the expansion of intensive operations with increased culture density. As a result, diseases occur more frequently, particularly during the early stages of production, which limits the sustainability of the aquaculture industry. Research on the relationships between nutrition and immunity has received increased attention in recent years (Trichet, 2010; Kiron, 2012). A growing body of evidence shows that dietary amino acids such as arginine (Arg) and glutamine (Gln) in particular, play a fundamental role in the overall immunity of fish (Buentello and Gatlin, 1999; Buentello et al., 2007; Cheng et al., 2011, 2012; Li et al., 2009; Wu, 2013).

Arginine is an essential amino acid for fish (Chen et al., 2012; Pohlenz et al., 2012a; Ren et al., 2012; Wei, 2010; Zhou et al., 2012a, b), not only as a precursor for protein synthesis but also for its metabolic role in the production of diverse metabolites, including nitric oxide (NO), polyamines, urea, proline, and glutamate (Jobgen et al., 2006; Li et al., 2009). It has been established that Arg plays an important role in the modulation of immune responses (Buentello et al., 2007; Jobgen et al., 2006). The main role of Arg in immune system is due to its catabolism catalyzed by inducible NO synthase (iNOS) into NO, which is an important immune mechanism against a wide range of pathogens (Bogdan et al., 2000). In addition, the arginase, which catalyzes the conversion of Arg to ornithine and urea, can also

regulate NO synthesis by modulating intracellular Arg availability (Morris, 1999) and by regulating iNOS expression (Lee et al., 2003).

Glutamine, an abundant free amino acid in fish plasma and muscle, is a preferred major fuel for rapidly dividing cells, including activated lymphocytes and intestinal epithelial cells (Wu et al., 1995; Calder and Yaqoob, 1999). Moreover, Gln plays important roles in eliminating free radicals (Amores-Sánchez and Medina, 1999) and is effective in protecting against H_2O_2-induced oxidative stress in the intestinal epithelial cells of carp (Chen et al., 2009). As a major energy substrate for leukocytes and a key modulator of cytokine and NO production, Gln is crucial to animal immune responses (Kim et al., 2007; Li et al. 2007). Gln is now considered as a conditionally essential amino acid (Li et al., 2009) and has been shown to be effective for improving growth in mammals (Tannuri et al., 2000; Bartell and Batal, 2007) and fish (Lin and Zhou, 2006; Xu et al., 2011).

The metabolism of Arg and Gln are closely related and play fundamental roles in enhancing immune function (Kim et al., 2007; Li et al., 2007). Dietary supplementation with Arg or Gln increases the expression of antioxidative genes and reduces the expression of proinflammatory genes in small intestine and adipose tissue (Jobgen et al. 2009; Wang et al. 2008). Buentello and Gatlin (1999) demonstrated that NO synthesis was increased when Arg and Gln were present in culture macrophages of channel catfish. In recent studies, dietary Arg and Gln improved nonspecific immune responses of red drum and hybrid striped bass (Cheng et al., 2011, 2012).

Turbot (*Scophthalmus maximus* L.) is an important commercial carnivorous fish species that has been widely farmed in Europe and East Asia because of its delicious meat and rapid growth. Studies of dietary Arg requirement and effects of dietary Arg on innate immune responses in turbot have been reported (Costas et al., 2013;

Fournier et al., 2003; Wei, 2010). However, there are no reports to date on the effects of dietary Gln on the growth performance of turbot. It is also not known whether dietary Arg and Gln have interactive effects on disease resistance and Arg catabolism in turbot. Thus, the present study was conducted to investigate the effects of dietary Arg, Gln, and their interactions on growth performance, nonspecific immune responses, disease resistance, and Arg catabolism in juvenile turbot.

2. Materials and methods

2.1. Experimental diets

L-Arg and L-Gln (>99% purity) were obtained from Hengyuan Biotech Co. (Shanghai, China). White fish meal, soybean meal, and corn gluten meal were used as the major protein sources; fish oil was used as the major lipid source; and wheat flour was used as the carbohydrate source. Lysine-H_2SO_4, DL-methionine, L-threonine, L-isoleucine, and L-valine (crystalline amino acids) were supplemented to meet the essential amino acid requirements of juvenile turbot based on the whole-body amino acid profile (Kaushik, 1998). Nine isoproteic (50% crude protein) and isolipidic (12% crude lipid) experimental diets were formulated with three levels of L-Arg and L-Gln supplementation (0.0%, 1.0%, and 2.0% each); L-glycine was adjusted to maintain equal nitrogen content among all the diets (Table 1). The amino acid composition of each experimental diet is shown in Table 2.

All ingredients were ground to a fine powder through a 180-μm mesh. Arg and Gln were blended into an amino acid premix. The ingredients were thoroughly mixed with fish oil, and water was added to produce a stiff dough. The dough was pelletized using an experimental feed mill (F-26[II], South China University of Technology, China), dried for approximately 12 h in a ventilated oven at 45°C, and stored at –20°C until use. No differences in physical quality or sinking properties were found among

the diets.

2.2. Fish, experimental conditions, and procedure

Juvenile turbot were obtained from Qingdao Tongyong Aquaculture Co. (Shandong, China). Prior to the start of the experiment, the fish were transported to the experimental station of the Ocean University of China (Qingdao), and stocked in an indoor recirculating seawater system (400-L circular fiberglass flat-bottom tanks filled to 300 L) for acclimatization to the experimental conditions for 2 weeks. During this period fish were fed twice daily with a commercial diet (Qihao Biotech Co. Shandong, China) to satiation. All rearing tanks were provided with continuous aeration and maintained under a natural photoperiod.

At the start of the experiment, fish were fasted for 24 h and weighed. Fish of similar size (initial body weight 4.64 ± 0.004 g) were randomly distributed among 27 tanks (35 fish per tank), and each of the nine experimental diets was assigned to three tanks. Fish were fed a fixed ration (initially 6% of body weight per day, gradually reduced to 2%) provided in two daily feedings (08:00 and 18:00 h) for 12 weeks. Fish were weighed every 2 weeks and the rations were adjusted accordingly. Throughout the experimental period, water temperature remained between 17.5 and 18.5°C; salinity was 30–33‰; pH was between 7.5 and 8.0; ammonia-nitrogen was <0.1 mg/L; nitrite was <0.1 mg/L; and dissolved oxygen was >6.0 mg/L.

2.3. Sample collection

At the termination of the trial, fish were fasted for 24 h and anesthetized with eugenol (1:10000) (purity 99%; Shanghai Reagent, China) before sampling. The total number and mean body weight of fish in each tank were measured. Six fish per tank were sampled for blood samples and liver samples for analysis. Blood samples were taken from the caudal vein using heparinized syringes to obtain serum after

centrifugation (for 10 min) at 4000 ×*g* and 4 °C. The serum samples were immediately frozen in liquid nitrogen and then stored at –80 °C until analysis. Liver samples were also immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

2.4. Chemical analysis

2.4.1. Dry matter, crude protein, crude lipid and ash assays

Dry matter, crude protein, crude lipid, and ash content in the dietary ingredients and experimental diets were analyzed (AOAC, 1995). Dry matter was determined by drying samples to a constant weight at 105 °C. Crude protein was determined using the Kjeldahl method and estimated by multiplying nitrogen content by 6.25. Crude lipid was quantified by Soxhlet extraction with ether. Ash content was quantified by combustion in a muffle furnace at 550 °C for 16 h. Duplicate analyses were conducted for each sample.

2.4.2. Amino acid determination

Amino acid in protein sources and experimental diets were determined according to the method of Zhang et al. (2013). Samples of experimental diets were freeze-dried and 0.02g of samples was used for amino acid analysis. The samples were hydrolyzed with 15 mL of 6 N HCl at 110 °C for 24 h, then filtered and added to ultrapure water (from Milli-Q system, Millipore, Billerica, MA, USA) in a 50 mL volumetric flask. A 2 mL solution was then transferred to a glass bottle and dried in a Binder Oven (VD23, Binder Company, Germany). Thereafter, 2 mL of ultrapure water was then added to the bottles and dried in the Binder Oven repeatedly three times, and then 2 mL of loading buffer was added to dissolve the remains. The supernatant was analysed by the ninhydrin method with an automatic amino acid analyzer (Biochrom 30, GE, Biochrom Ltd, Cambridge, UK), equipped with a sodium exchange column (μ-2345).

The column temperature was 37-135 °C. Ultraviolet detection was performed at a wavelength of 440 nm (for proline) and 570 nm (for other amino acids).

2.4.3 Respiratory burst activity assay

Head-kidney macrophage suspensions were isolated from three fish in each tank according to Secombes et al. (1990) with modifications. Respiratory burst activity produced by the head-kidneys macrophages was assayed by measuring the reduction of nitroblue tetrazolium (NBT; Sigma) according to Secombes et al. (1990) with modifications. A 100 μl cell suspension was stained with 200 μl 0.2% NBT and 200 μl phorbol 12-myristate 13-acetate (PMA; 1 mg/ml; Sigma) which was used as a trigger for O^{2-} production for 40 min. Absolute methanol was added to terminate the staining. Each tube was washed twice times with 70% methanol and air-dried. Then, 120 ml 2m-KOH and 140 ml dimethyl sulfoxide (DMSO) were added and absorbance was measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank.

2.4.4 Lysozyme activity assay

Lysozyme activity in serum was measured according to Ellis (1990). Briefly, a 0.01 mL sample of serum was added to 1.4 mL of a 0.2 mg/mL suspension of *Micrococcus lysodeikticus* (Sigma) in 0.1 M sodium phosphate buffer (pH 6.8). The reaction was carried out at 25 °C and absorbance was measured in a spectrophotometer at 530 nm after 0.5 and 4.5 min. One unit was defined as the amount of sample that caused a decrease in absorbance of 0.001 per min.

2.4.5 Preparation of liver supernatants and protein content assay

Liver samples in each tank were weighed and homogenized in ice-cold 0.7% saltwater (1:9 w/v). Following centrifugation at 3000 r/min for 10 min, the supernatants were collected and maintained at –80 °C for analysis of protein concentration, catalase (CAT), and iNOS activities. Protein concentrations of liver

supernatants were measured according to the analytical procedures specified in a total protein quantitative assay kit (Bradford) using bovine serum albumin as standard (Nanjing Jiancheng Bioengineering Institute, China).

2.4.6 Antioxidant-related enzyme activity assay

Superoxide dismutase (SOD) and glutathione-peroxidase (GPx) activities in serum, and CAT activities in liver were measured with a spectrophotometer at 450, 412, and 405 nm, respectively, following the kit instructions (Nanjing Jiancheng Bioengineering Institute). One unit of SOD activity was defined as the amount of enzyme required to inhibit superoxide-induced oxidation by 50%. One unit of GPx activity was defined as the amount of enzyme that reduced the glutathione concentration in the reaction system by 1 μmol/L per min. One unit of CAT activity was defined as the amount of enzyme that catalyzed the decomposition of 1 μmol of H_2O_2 per min per mg protein.

2.4.7. iNOS activity assay

iNOS activity was determined by its ability to convert L-Arg to NO, using an NOS Kit (Nanjing Jiancheng Bioengineering Institute). NO could be oxidized to nitrite or nitrate. Nitrite was then detected by Griess assay reagents [sulfanilamide, H_3PO_4, and *N*-(1-naphthyl)-ethylendi-amine (NED)]. Optical density was measured at 530 nm following the manufacturer's instructions. One unit of iNOS activity was defined as the amount of iNOS that produced 1 nmol NO per min. The iNOS activity was expressed as units of iNOS per mL serum or per mg protein in liver supernatants.

2.4.8. Real-time quantitative PCR analysis of arginase I gene expression

Total RNA was extracted from liver samples using Trizol Reagent (Invitrogen, USA), and electrophoresed on a 1.2% denaturing agarose gel to check integrity. The RNA was treated with Recombinant DNase I (RNase-free) (Takara, Japan) to remove

possible DNA contamination according to the manufacturer's instructions. The quantity and quality of total RNA were assessed using the Nano Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratios of all samples ranged from 1.98 to 2.06, indicating satisfactory RNA purity. Purified RNA was subjected to reverse transcription to cDNA using a PrimeScriptTM RT reagent kit (Takara, Japan) according to the manufacturer's instructions. The real-time PCR primers were designed using Primer Premier 5.0 based on the nucleotide sequences of turbot arginase I gene (GenBank accession number: KF591747). Real-time PCR assays were carried out using a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany) in a final volume of 25 μl containing 12.5 μl 2 × SYBR® Premix Ex TaqTM (Perfect Real Time) (Takara, Japan), 1 μl each primer (10 μM), and 2μl cDNA mix. The arginase I gene-specific primers arginase I F (5'-GTGTTCTCCATGTCGCAGGTG-3') and arginase I R (5'-GGGCAACAGACGGGTCAATG-3') were applied to evaluate mRNA levels of arginase I gene in liver samples. Reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (DQ848904) (F: 5'-TCCAATGTTTGTCATGGGAGTT-3'; R: 5'-CCAGAGGAGCCAGGCAGTT-3') was used as an internal control. The real-time PCR amplification began with 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C. No template controls were run for each PCR assay. A five-fold serial dilution was used to assess PCR efficiency for each assay by quantifying five concentrations (in triplicate). Primer amplification efficiency was analyzed using following equation: $E=10(-1/Slope)-1$. The efficiency value was 1.013 for arginase I and 1.034 for GAPDH. The absolute ΔCT value (arginase I CT − GAPDH CT) of the slope was 0.046, which indicated that the ΔΔCT calculation for the relative quantification of

arginase I gene could be used. The expression level of arginase I gene was calculated using the 2-ΔΔCT method, and the expression value represented the *n*-fold difference relative to the calibration (Livak and Schmittgen, 2001).

2.4.9. Challenge experiment

Edwardsiella tarda was purchased from the College of Marine Life Science, Ocean University of China (Qingdao) and was activated following the methods described by Alexander et al. (2011). The 7-d median lethal dose (LD_{50}) was determined by intraperitoneal injection of 15 fish (similar in size to the experimental fish) with graded doses of *E. tarda* (1.44×10^5, 1.44×10^6, 1.44×10^7 and 1.44×10^8 cfu/mL) at 20 °C; the LD_{50} on day 7 was 1.44×10^6 cfu/mL.

After conducting the feeding trials and sampling, the fish were feeding the same diets as provided during the feeding trial twice daily for 7 d to acclimatization to the experimental conditions. Then, 15 fish from each tank were injected with 0.2 mL phosphate-buffered saline containing 1.44×10^6 cfu/mL *E. tarda* using medical syringes. Fish were continued feeding the same diets as before after injection. Fish were carefully monitored and mortality was recorded five times each day for the next 7 d according to Deng et al. (2012) and Liu et al. (2014). Survival rate (SR) was determined on the seventh day as follows:

SR (%) = 100 × final fish number / initial fish number

2.5. Calculations and statistical methods

The following variables were calculated:

Survival rate (SR %) = 100 × final fish number / initial fish number

Relative growth rate (RGR %/day) = $(e.^g - 1)*100$, with g = (Ln final body weight – Ln initial body weight) / days.

Feed intake (FI %/d) = 100 × dry feed intake (g) / [(final body weight + initial body

weight) / 2] / days

Feed conversion rate (FCR) = wet weight gain (g) / dry feed intake (g)

Protein efficiency ratio (PER) = wet weight gain (g) / protein ingested (g)

All statistical analyses were performed using SPSS 17.0. Factorial (two-way) analysis of variance (ANOVA) was used to determine the main effects of dietary Arg and Gln levels, and their interactions on growth performance, nonspecific immune parameters, disease resistance, and expression of arginase I gene. When significant interaction and significant main effects of dietary Arg and Gln levels were observed at the same time, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among all the treatments. When only a significant interaction and significant main effects of dietary Arg or Gln levels were observed, data were analyzed by one-way ANOVA followed by Tukey's multiple range tests to inspect differences among Gln levels within each level of Arg, and vice versa. When the significance is only with the main effects of Arg or Gln level, the data were analyzed by the two-way ANOVA followed by Tukey's multiple range tests to assess the main effects of dietary Arg or Gln only. Arginase I gene expression and survival after disease challenge were also subjected to linear regression analysis against dietary Arg or Gln level. Percentage data were arcsine transformed before statistical analysis. Differences were regarded as significant at $P<0.05$. All data are presented as the means of three replications.

3. Results

3.1. Survival rate and growth performance

The SR of juvenile turbot increased significantly as dietary Arg increased ($P=0.005$), However, Gln supplementation had no significant effects on SR ($P=0.283$) and no significant interactions between dietary Arg and Gln were found ($P=0.225$).

There were no significant differences in RGR (2.37–2.44 %/d, P=0.722), FCR (1.11–1.14, P=0.814), or PER (2.20–2.27, P=0.580) in juvenile turbot fed diets with different Arg and Gln levels (Table 3).

3.2. Nonspecific immune parameters

Respiratory burst activity in head-kidney macrophages increased significantly as dietary Arg or Gln increased (P<0.001, P<0.001), but no significant interactions between dietary Arg and Gln were found (P=0.512, Table 4). Lysozyme activity in serum was significantly increased as dietary Arg increased only at low Gln (0.0%) group (P=0.003) and significantly increased as dietary Gln increased only at low Arg (0.0%) group (P=0.004, Table 5). GPx activity in serum was significantly increased as dietary Arg increased only in the treatment at Gln supplementation level of 0.0% (P=0.017) and 2.0% (P=0.032) and significantly increased as dietary Gln increased only at low Arg (0.0%) group (P=0.046). Hepatic CAT activity increased significantly as dietary Arg increased (P=0.002). Neither dietary Gln nor its interactions with Arg significantly affected hepatic CAT activity (P=0.612, P=0.226). SOD activity in serum was independent of dietary Arg and Gln levels (P=0.227).

3.3 iNOS activity in serum and liver

Significant interactions between dietary Arg and Gln occurred in relation to iNOS activity in serum and liver (P=0.041, P<0.001, Table 6). iNOS activity in liver increased significantly as dietary Arg or Gln increased in the treatments at Gln or Arg supplementation level of 0.0%, but significantly decreased as dietary Arg or Gln increased in the treatments at Gln or Arg supplementation level of 1.0 and 2.0% (P<0.001, P=0.023), respectively.

3.4. Expression of arginase I gene in liver

Expression of the arginase I gene in liver tissue of fish was significantly affected

by interactions between dietary Arg and Gln (P=0.006, Fig.1). Arginase I gene expression in liver tissue of fish were significantly enhanced as dietary Arg and Gln increased (P=0.001, P<0.001)，and significantly correlated with dietary Arg/Gln level (P=0.017, P=0.001 Table 7). Fish fed the diet with the highest Arg and Gln supplementation level (2.0%) showed the highest level of arginase I gene expression (1.82, Fig.1).

3.5 Challenge test

Post-challenge survival rates of juvenile turbot were significantly improved as dietary Arg increased (P=0.005, Table 4), and significantly correlated with dietary Arg level (P<0.001, Table 7). However, no significant effects of dietary Gln or its interactions with Arg were observed (P=0.283, P=0.225).

4. Discussion

The requirement for dietary Arg for optimal growth in fish has been confirmed by many studies (Chen et al., 2012; Pohlenz et al., 2012a; Ren et al., 2012; Zhou et al., 2012a, b). In the present study, dietary Arg supplementation did not have a significant effect on growth performance of juvenile turbot, but significantly enhanced SR. This indicated that dietary supplemental of 1.0% Arg (3.00% of diet) might be necessary for maintaining normal survival of juvenile turbot. This finding is consistent with that of Fournier et al. (2003), who recommended that the Arg requirement for juvenile turbot (7.4 g) is between 1.6% and 3.0% of dry diet. Furthermore, these results were similarly to the reported requirement of 3.13% Arg for turbot (initial body weight 1.80 g) (Wei, 2010).

Dietary supplementation with Gln had no significant effect on the growth of juvenile turbot in the present study. Similarly, limited effects of dietary Gln supplementation on growth have been reported for channel catfish (Pohlenz et al.,

2012b), large yellow croaker (Gao et al., 2010), Nile tilapia (Yang, et al., 2008), and red drum (Cheng et al., 2011), as well as for piglets (House et al., 1994), dairy cattle (Plaizier et al., 2001) and broiler chickens (Murakami et al., 2007). However, these findings are inconsistent with the studies for Jian carp (Lin and Zhou, 2006), hybrid striped bass (Cheng et al., 2012), and hybrid sturgeon (Xu et al., 2011), in which dietary Gln supplementation enhanced growth performance. Important physiological differences in Gln metabolism might exist among different species (Cheng et al., 2011; Pohlenz et al., 2012b), and these inconsistencies may be due to the differences of experimental animals. In addition, developmental stage, environmental factors, and physiological or pathological state may affect Gln utilization.

Many studies have demonstrated the important regulatory roles for Arg and Gln in enhancing immune function in animals (Kim et al., 2007; Li et al., 2007). In fish, the nonspecific immune system provides important protection against pathogens (Siwicki et al., 1994). Production of superoxide anion is considered to be one of the most important antimicrobial mechanisms in phagocytes (Secombes, 1990). In this study, respiratory burst activity in head-kidney macrophages increased significantly as dietary Arg or Gln increased, which indicated that superoxide anion production could be increased with the increase of dietary Arg and Gln. Lysozyme is an opsonin that can lyse pathogens, activate and complement the activity of phagocytes (Magnadottir, 2010). Lysozyme activity was significantly increased as dietary Arg increased at low Gln (0.0%) group and significantly increased as dietary Gln increased at low Arg (0.0%) group. The significant increase of lysozyme activity in serum of juvenile turbot fed diet with increased Arg and Gln was in agreement with findings for hybrid striped bass and red drum (Cheng et al., 2011, 2012). These results were also consistent with the findings of Costas et al. (2011) and Costas et al. (2013), which

showed that respiratory burst activity and plasma lysozyme activity were significantly enhanced as dietary Arg supplementation.

High levels of reactive oxygen species can oxidize cell lipid constituents and therefore threaten cell integrity. Antioxidant enzymes, including GPx, CAT, and SOD, provide the first line of enzymatic defense against free radicals (Deng et al., 2012). Arg and Gln are precursors for the synthesis of antioxidant polyamines (Igarashi and Kashiwagi, 2000) and glutathione (Tovar-Ramirez et al., 2010), which exert beneficial effects on peroxidation protection. In the present study, GPx activity in serum was significantly increased as dietary Arg increased at Gln supplementation level of 0.0% and 2.0% and significantly increased as dietary Gln increased at Arg supplementation level of 0.0%. Hepatic CAT activity was significantly increased as dietary Arg increased. These results indicated that dietary Arg and Gln supplementation could improve antioxidant capacity in turbot. Antioxidative effects of Arg and Gln were also observed in studies conducted in other fish species (Buentello et al., 2007; Chen et al., 2009), rats (El-sheikh and Khalil, 2011), and pigs (Ma et al., 2010).

Results of the present study highlighted the potent immune functions of Arg and Gln in fish, which is consistent with other findings (Cheng et al., 2011; 2012; Pohlenz et al., 2012c). Arg and Gln may enhance immune responses via a pathway that involves NO synthesis (Costas et al., 2011; 2013; Moinard et al., 2002). Supplementation of culture media with Arg and/or Gln enhanced NO production by activated macrophages of channel catfish (Buentello and Gatlin, 1999; 2001). iNOS was the rate-limiting enzyme during NO production (Bogdan et al., 2000). In this study, iNOS activity in liver was significantly increased with dietary Arg and Gln increased at Gln or Arg supplementation level of 0.0%. The beneficial effects of Arg and Gln on immune response could be due to the increased NO production catalyzed

by increased iNOS activity. The possible mechanism may be due to the fact that Arg functions as the substrate for iNOS and Gln provides metabolic fuel for NO production. However, it should be noted that while physiological levels of NO play an important role in cellular signaling, excess NO produced by iNOS can result in oxidative injury and apoptosis (Jobgen et al. 2006). The exact mechanism underlying the effects of NO on immune functions is not clear and warrants further investigation.

The arginase pathway, quantitatively most important for the catabolism of Arg (Durante et al., 2007), is also critical for its role in immunity (Evoy et al., 1998). In the present study, arginase I gene expression in the liver of fish were significantly correlated with dietary Arg and Gln levels and fish fed the diet with the highest Arg and Gln level showed the highest level of arginase I gene expression. It could be concluded that to some extent the activity of arginase I in liver was enhanced as dietary Arg and/or Gln increased. This is in agreement with the findings of Berge et al. (1997) and Tulli et al. (2007), who observed that hepatic arginase activity was significantly increased as dietary Arg increased. Modifications in the expression and activity of arginase may affect NO generation (Gobert et al., 2001; Morris, 1999) as a result of competition between arginase and iNOS for Arg and by regulating iNOS expression (Lee et al., 2003).

In the present study, dietary Arg supplementation significantly enhanced SR of juvenile turbot after a 7-day challenge with *E. tarda*, which was consistent with the enhanced nonspecific immune responses. Similarly, dietary Arg supplementation enhanced the immune functions of fish during immunological challenge (Buentello and Gatlin, 2001; Calder and Yaqoob, 2004; Costas et al., 2011; 2013; Pohlenz et al., 2012d). However, dietary Gln supplementation had no significant effects on SR of juvenile turbot after *E. tarda* infection in this study, which was inconsistent with the

findings for hybrid tilapia that showed significant enhancement of SR after 48 h or 72 h challenge with *Aeromonas hydrophila* by dietary Gln supplementation (Yang et al., 2008). This could be due to the different fish species and sampling time. In this study, immunological parameters were just assayed at the end of the feeding trail. Following studies should be focused on the detection and comparison of immune responses before and after challenge test to obtain a better understanding of the related mechanisms.

In conclusion, results from the present study showed that dietary inclusion of Arg and Gln did not have a positive effect on growth performance of juvenile turbot, but significantly enhanced nonspecific immune responses. Dietary Arg and Gln levels significantly affected Arg catabolism in juvenile turbot. Disease resistance of juvenile turbot was significantly improved with the increasing of dietary Arg. Based on these results, additional supplementation of 1.0% Arg in the diet was positive for the immune and disease resistance of turbot. Further research is still needed to elucidate the molecular mechanisms of Arg and Gln on immune responses. It is also necessary to validate the use of Arg and Gln as suitable alternatives for disease control in the culture of turbot.

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Figure captions

Fig. 1. Relative mRNA expression of arginase I gene in liver of juvenile turbot fed diets with different arginine/glutamine levels for 12 weeks. Relative mRNA expression was evaluated by real-time quantitative PCR. Each data represents the mean of three replicates. Bars assigned with the same superscripts are not significantly different ($P>0.05$). Two-way ANOVA showed the following P values: Arginine, $P=0.001$; Glutamine, $P<0.001$; Interaction, $P=0.006$.

Fig. 1.

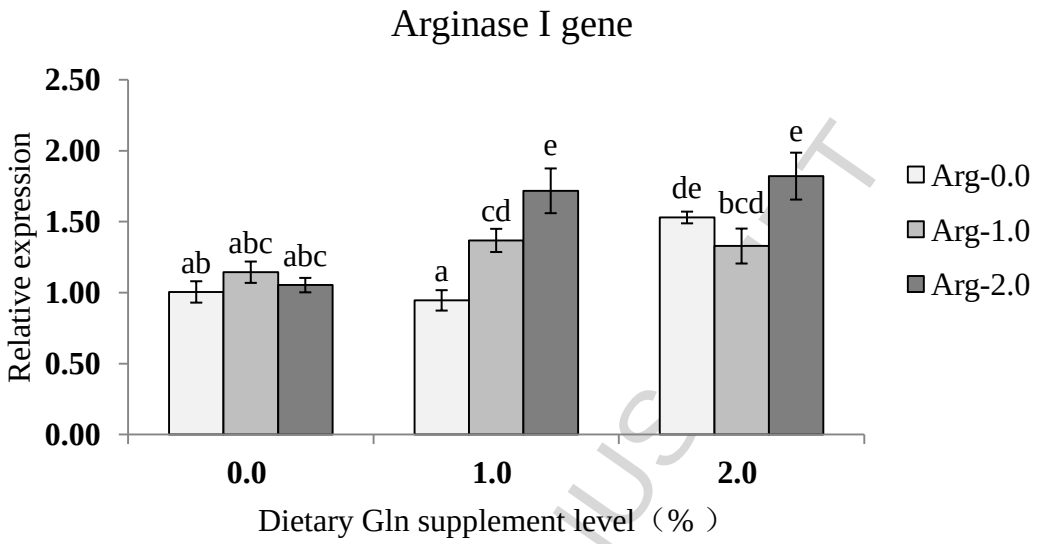

Table 1
Formulation and chemical proximate composition of the experimental diets (% dry matter)

Ingredients	Arginine/Glutamine supplement level (% dry matter)								
	0.0/0.0	0.0/1.0	0.0/2.0	1.0/0.0	1.0/1.0	1.0/2.0	2.0/0.0	2.0/1.0	2.0/2.0
White fish meal[a]	23	23	23	23	23	23	23	23	23
Soybean meal[a]	16	16	16	16	16	16	16	16	16
Corn gluten meal[a]	23	23	23	23	23	23	23	23	23
	14.27	14.27	14.27	14.27	14.27	14.27	14.27	14.27	14.27
Wheat flour	7	7	7	7	7	7	7	7	7
Fish oil	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
$Ca(H_2PO_3)_2$	2	2	2	2	2	2	2	2	2
Soybean lecithin	2	2	2	2	2	2	2	2	2
Choline（99%）	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Amino acid mixture[b]	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Vitamin premix[c]	2	2	2	2	2	2	2	2	2
Mineral premix[d]	1	1	1	1	1	1	1	1	1
Attractant[e]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	4	3	2	3	2	1	2	1	0
Arginine[f]	0	0	0	1	1	1	2	2	2
Glutamine[f]	0	1	2	0	1	2	0	1	2
Proximate analysis (%, on a dry weight basis)									
Crude protein	50.85	50.46	50.40	50.86	50.48	50.36	51.08	50.52	50.41
Crude lipid	12.26	12.31	12.67	12.44	12.60	12.24	11.98	12.01	12.55

[a] Supplied by Qihao Biotech. Co., Ltd. (Shandong, China); white fish meal, crude protein, 71.18%, crude lipid, 6.17%; soybean meal, crude protein, 51.53%, crude lipid 1.13%; corn gluten meal, crude protein, 61.61%, crude lipid 3.78%.

[b] Amino acid premix (g kg^{-1} diet): Lys-H_2SO_4, 5; DL-methionine, 4; L-threonine, 3; L-isoleucine, 2; L-valine, 5; L-alanine, 10; L-aspartic acid, 8; L-serine, 5; L-tyrosine, 4.

[c] Vitamin premix (mg kg^{-1} diet): retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B_{12} (1%), 10; L-ascorbyl-2-monophosphate-Na (35%),

2000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; ethoxyquin, 503; cellulose, 15970.

[d] Mineral premix (mg kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 1200; $CuSO_4 \cdot 5H_2O$, 10; $ZnSO_4 \cdot H_2O$, 50; $FeSO_4 \cdot H_2O$, 80; $MnSO_4 \cdot H_2O$, 45; CoCl(1%), 50; Na_2SeO_3(1%), 20; $Ca(IO_3)_2$(1%), 60; calcium propionate, 1000; zoelite, 7485.

[e] Attractants: taurine : glycine : betaine =1 : 3 : 3.

[f] L-Arginine and L-Glutamine were obtained from Hengyuan Biotech. Co., Ltd (Shanghai, China).

Table 2
Amino acid composition of the experimental diets (% dry matter)

Ingredients	Arginine/Glutamine supplement level (% dry matter)								
	0.0/0.0	0.0/1.0	0.0/2.0	1.0/0.0	1.0/1.0	1.0/2.0	2.0/0.0	2.0/1.0	2.0/2.0
Asx[1]	4.17	4.20	4.20	4.14	4.09	4.19	4.18	4.12	4.17
Threonine	1.80	1.80	1.81	1.77	1.77	1.79	1.79	1.76	1.79
Serine	2.37	2.38	2.40	2.37	2.35	2.37	2.38	2.32	2.34
Glx[2]	6.91	7.73	8.57	6.86	7.53	8.47	6.79	7.44	8.44
Proline	2.47	2.44	2.51	2.40	2.42	2.42	2.54	2.53	2.55
Glycine	6.87	5.85	4.74	5.62	4.77	3.68	4.76	3.93	2.59
Alanine	3.24	3.27	3.30	3.25	3.34	3.39	3.38	3.33	3.30
Valine	2.52	2.54	2.59	2.44	2.48	2.48	2.44	2.40	2.50
Isoleucine	1.86	1.88	1.89	1.79	1.82	1.84	1.82	1.78	1.83
Leucine	4.66	4.72	4.79	4.58	4.62	4.65	4.71	4.48	4.66
Tyrosine	1.75	1.80	1.85	1.73	1.74	1.70	1.73	1.66	1.77
Phenylalanine	2.04	2.07	2.10	1.99	2.01	2.02	2.03	2.02	2.10
Histidine	0.85	0.86	0.86	0.83	0.84	0.84	0.83	0.83	0.84
Lysine	2.47	2.46	2.44	2.37	2.38	2.42	2.43	2.43	2.39
Arginine	2.10	2.13	2.07	2.97	3.00	3.02	3.86	3.82	3.87

[1]Asx means aspartic acid (Asp) +asparagine (Asn).

[2]Glx means glutamic acid (Glu)+ glutamine (Gln).

Table 3
Survival rate and growth performance of juvenile turbot fed diets with different arginine/glutamine levels (n = 3)[1]

Arginine supplement level (%)	Glutamine supplement level (%)	IBW[2] (g)	FBW[3] (g)	RGR (%/d)[4]	FCR[5]	PER[6]	SR[7] (%)
		Individual treatment means					
0.0	0.0	4.63	31.55	2.42	1.14	2.23	93.33
0.0	1.0	4.62	31.33	2.42	1.12	2.21	96.19
0.0	2.0	4.65	31.53	2.42	1.12	2.23	98.10
1.0	0.0	4.64	30.71	2.39	1.13	2.22	99.05
1.0	1.0	4.64	31.19	2.41	1.14	2.27	100.00
1.0	2.0	4.64	30.43	2.37	1.11	2.20	99.05
2.0	0.0	4.63	32.14	2.44	1.14	2.23	99.05
2.0	1.0	4.64	31.27	2.41	1.12	2.24	98.10
2.0	2.0	4.66	30.93	2.39	1.11	2.22	99.05
Pooled SE		0.004	0.179	0.007	0.006	0.008	0.502
		Means of main effect					
0.0		4.63	31.47	2.42	1.13	2.22	95.87[a]
1.0		4.64	30.78	2.39	1.13	2.23	98.73[b]
2.0		4.64	31.45	2.42	1.12	2.23	99.37[b]
	0.0	4.63	31.47	2.42	1.13	2.23	97.14
	1.0	4.63	31.26	2.41	1.13	2.24	98.10
	2.0	4.65	30.96	2.40	1.11	2.22	98.73
Two-way ANOVA: *P*-values[8]							
Arginine		0.749	0.242	0.282	0.980	0.983	0.005
Glutamine		0.233	0.541	0.407	0.442	0.634	0.283
Arginine × Glutamine		0.789	0.672	0.722	0.814	0.580	0.225

[1] Values in the same column with the same superscript or absence of superscript are not significant different (P>0.05).

[2] IBW: initial body weight; [3] FBW: final body weight.

[4] RGR: relative growth rate (%/day) = (e^{g} - 1)*100, with g = (Ln FBW – Ln IBW) / days.

[5] FCR: feed conversion rate = wet weight gain (g) / dry feed intake (g).

[6] PER: protein efficiency ratio = wet weight gain (g) / protein ingested (g).

[7] SR: survival rate = 100 × final fish number / initial fish number.

[8] Differences were regarded as significant when $P<0.05$. Arginine and glutamine showed the main effect of each factor, and arginine × glutamine indicated their interactive effect.

Table 4
Nonspecific immune parameters of juvenile turbot fed diets with different arginine/glutamine levels (n = 3)[1]

Arginine supplement level (%)	Glutamine supplement level (%)	Respiratory burst activity (OD 10^{-7} cell)	Serum SOD activity (U ml^{-1})	Liver CAT activity (U/mg prot)	Post-challenge SR (%)
		Individual treatment means			
0.0	0.0	0.137	23.03	8.76	44.44
0.0	1.0	0.236	22.53	8.32	48.89
0.0	2.0	0.265	25.06	7.31	46.67
1.0	0.0	0.216	19.91	10.52	55.56
1.0	1.0	0.326	24.64	9.87	51.11
1.0	2.0	0.303	21.47	11.37	66.67
2.0	0.0	0.331	22.28	9.19	75.56
2.0	1.0	0.436	23.35	9.56	77.78
2.0	2.0	0.388	24.08	10.81	82.22
Pooled SE		0.018	0.467	0.308	3.236
		Means of main effect			
0.0		0.213[a]	23.54	8.13[a]	46.67[a]
1.0		0.282[b]	22.01	10.59[b]	57.78[a]
2.0		0.385[c]	23.23	9.85[b]	78.52[b]
	0.0	0.228[x]	21.74	9.49	58.52
	1.0	0.333[y]	23.51	9.25	59.26
	2.0	0.319[y]	23.54	9.83	65.19
Two-way ANOVA: *P*-values[2]					

Arginine	<0.001	0.332	0.002	0.005
Glutamine	<0.001	0.179	0.612	0.283
Arginine × Glutamine	0.512	0.227	0.226	0.225

[1] Values in the same column with the same superscript or absence of superscript are not significant different (P>0.05).

[2] Differences were regarded as significant when P<0.05. Arginine and glutamine showed the main effect of each factor, and arginine × glutamine indicated their interactive effect.

Table 5
Lysozyme and GPx activity in serum of juvenile turbot fed diets with different arginine/glutamine levels (n = 3)[1]

Arginine supplement level (%)	Glutamine supplement level (%)	Serum lysozyme activity (ug ml^{-1})	Serum GPx activity (U ml^{-1})
		Individual treatment means	
0.00	0.00	180.67[a]	39.22[a]
0.00	1.00	254.67[b]	56.39[b]
0.00	2.00	255.67[b]	48.72[ab]
1.00	0.00	219.33	49.66
1.00	1.00	233.00	47.38
1.00	2.00	232.67	48.79
2.00	0.00	236.67	56.99
2.00	1.00	246.33	49.45
2.00	2.00	221.00	58.91
0.00	0.00	180.67[a]	39.22[a]
1.00	0.00	219.33[b]	49.66[ab]
2.00	0.00	236.67[b]	56.99[b]
0.00	1.00	254.67	56.39
1.00	1.00	233.00	47.38
2.00	1.00	246.33	49.45
0.00	2.00	255.67	48.72[a]
1.00	2.00	232.67	48.79[a]
2.00	2.00	221.00	58.91[b]
Pooled SE		4.800	1.524
		Means of main effect	
0.00		230.33	48.11[a]
1.00		228.33	48.61[a]
2.00		234.67	55.12[b]
	0.00	212.22[a]	48.62
	1.00	244.67[b]	51.07
	2.00	236.44[b]	52.14
Two-way ANOVA: *P*-values[2]			
Arginine level		0.627	0.048
Glutamine level		< 0.001	0.509
Arginine level × Glutamine level		< 0.001	0.036

[1] Values in the same column with the same superscript or absence of superscript are not significant different (P>0.05).

[2] Differences were regarded as significant when $P<0.05$. Arginine and glutamine showed the main effect of each factor, and arginine × glutamine indicated their interactive effect.

Table 6
iNOS activity of juvenile turbot fed diets with different arginine/glutamine levels (n = 3)[1]

Arginine supplement level (%)	Glutamine supplement level (%)	Serum iNOS activity (U/ml)	Liver iNOS activity (U/mg prot)
		Individual treatment means	
0.0	0.0	6.47	2.99^{b}
0.0	1.0	6.14	3.01^{b}
0.0	2.0	7.57	4.40^{c}
1.0	0.0	5.01	2.06^{ab}
1.0	1.0	7.35	1.92^{a}
1.0	2.0	6.65	1.42^{a}
2.0	0.0	6.29	4.39^{c}
2.0	1.0	6.27	2.18^{ab}
2.0	2.0	5.81	1.93^{a}
Pooled SE		0.199	0.220
		Means of main effect	
0.0		6.73	3.47
1.0		6.34	1.80
2.0		6.13	2.83
	0.0	5.93	3.15
	1.0	6.59	2.37
	2.0	6.68	2.59
Two-way ANOVA:*P*-values [2]			
Arginine		0.355	<0.001
Glutamine		0.167	0.023
Arginine × Glutamine		0.042	<0.001

[1] Values in the same column with the same superscript or absence of superscript are not significant different (P>0.05).

[2] Differences were regarded as significant when P<0.05. Arginine and glutamine showed the main effect of each factor, and arginine × glutamine indicated their interactive effect.

Table 7
Correlation coefficients (r) and *P* values of dietary Arg or Gln levels (X) vs. Arginase I gene expression and survival rate after disease challenge [1].

	Arginine level (%)	Glutamine level (%)	Linear regression	r	*P*
Arginase I gene expression		0.00	$Y=0.028X+0.981$	0.354	0.639
		1.00	$Y=0.441X+0.026$	0.999	0.001
		2.00	$Y=0.163X+1.072$	0.576	0.236
		total	$Y=0.211X+0.693$	0.977	0.017
	0.00		$Y=0.262X+0.897$	0.816	0.017
	1.00		$Y=0.092X+1.187$	0.771	0.217
	2.00		$Y=0.384X+1.146$	0.921	0.008
	total		$Y=0.246X+1.077$	0.997	0.001
Survival rate after disease challenge		0.00	$Y=17.73X+5.637$	0.984	0.011
		1.00	$Y=16.38X+10.38$	0.892	0.041
		2.00	$Y=20.33X+4.543$	0.998	<0.001
		total	$Y=15.92X+29.13$	0.985	<0.001
	0.00		$Y=1.111X+45.55$	0.500	0.801
	1.00		$Y=2.222X+48.88$	0.500	0.197
	2.00		$Y=3.333X+75.18$	0.982	0.514
	total		$Y=3.333X+57.65$	0.912	0.414

[1] Differences were regarded as significant when $P<0.05$.

Highlights

- Dietary inclusion of Arg and Gln had no positive effect on growth performance.
- Increased dietary Arg and/or Gln improved nonspecific immune responses.
- Dietary Arg and Gln levels significantly affected Arg catabolism.
- Disease resistance of turbot was significantly enhanced as dietary Arg increased.

Statement of Relevance

The present study mainly investigated effects of dietary arginine (Arg), glutamine (Gln), and their interactions on growth performance, nonspecific immunity, disease resistance, and Arg catabolism in juvenile turbot (*Scophthalmus maximus* L.). Our findings have showed that dietary inclusion of Arg and Gln have no positive effect on growth performance of juvenile turbot, but significantly improved nonspecific immune responses. Dietary Arg and Gln levels had significantly interactive effects on Arg catabolism. Disease resistance was significantly enhanced with the increasing of dietary Arg. The results are reliable and of both theoretical and practical importance.

The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed. I have read and have abided by the statement of ethical standards for manuscripts submitted to Aquaculture.