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Dietary arginine affects growth, gut morphology, oxidation resistance and immunity of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) juveniles

Mingjuan Wu[1,2,3], Xiaoyi Wu[1,2,3]*, Senda Lu[1,2,3], Yujie Gao[1,2,3], Wei Yao[1,2,3], Xiaojun Li[1,2,3], Yu Dong[1,2,3] and Zibo Jin[1,2,3]

[1]*State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou 570228, People's Republic of China*
[2]*Department of Aquaculture, Ocean College of Hainan University, Haikou, 570228, People's Republic of China*
[3]*Hainan Provincial Key Laboratory for Tropical Hydrobiology and Biotechnology, Department of Aquaculture, Hainan University, Haikou, Hainan 570228, People's Republic of China*

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Abstract

An 8-week growth trial was conducted to evaluate the effects of dietary arginine (Arg) levels on growth, gut morphology, oxidation resistance and immunity of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) juveniles. Seven isoenergetic (1465 kJ (350 kcal)/100-g DM), isoproteic (53·5 % of DM) and isolipidic (7 % of DM) experimental diets were formulated to contain graded Arg levels ranging from 1·9 to 4·7 % (dry weight) at approximately 0·5 % increments. Each diet was randomly assigned to triplicate groups of 16 juvenile fish (average initial body weight: 11·7 (SD 0·1) g) and was administered twice daily (08.00 and 16.00 hours). After the growth trial, all remaining fish were fed their prescribed diets for 2 d and then exposed to 4·5 mg Cu^{2+}/l water for 36 h. Results showed that growth performance and feed utilisation of experimental fish were significantly affected by different dietary Arg levels. Weight gain % (WG%) of fish was increased as dietary Arg increased, reaching a peak value at 3·8 % dietary Arg level, and when dietary Arg level increased to 4·7 % WG% was reduced. Fish fed 1·9 and 2·2 % dietary Arg levels had higher daily feed intake compared with fish fed other dietary Arg levels. Feed conversion ratios in fish fed 1·9, 2·2, 2·7 and 4·7 % dietary Arg levels were higher than those in fish fed 3·1, 3·8 and 4·1 % dietary Arg levels. Protein efficiency ratio and protein productive value (PPV) increased with an increase in dietary Arg, up to a peak value at 3·8 % dietary Arg level, above which these parameters declined. On the basis of quadratic regression analysis of weight gain % (WG%) or PPV against dietary Arg levels, the optimal dietary Arg requirement for hybrid grouper was estimated to be 3·65 %. Fish fed 3·8 % dietary Arg had higher whole-body and muscle protein contents compared with fish fed other dietary Arg levels. Fish fed 3·8 and 4·1 % dietary Arg levels had higher levels of mRNA for insulin-like growth factor-I and target of rapamycin in the liver compared with fish fed other dietary Arg levels. Hepatic S6 kinase 1 mRNA expression in fish fed 3·8 % dietary Arg level was higher than that in fish fed any of the other dietary Arg levels. Gut morphology, hepatic antioxidant indices and immune indices in serum and head kidney were significantly influenced by dietary Arg levels. In conclusion, the optimal dietary Arg requirement for hybrid grouper was estimated to be 3·65 %, and suitable dietary Arg supplementations improved gut morphology and oxidation resistance of hybrid grouper.

Key words: **Hybrid grouper: Arginine: Growth: Immunity**

Arginine (Arg) is not only an essential amino acid in all fish species studied so far[1] but also a functional amino acid as reported in many studies on Arg nutrition of fish[2–5]. Dietary Arg requirements have been established for many commercial fish species, such as channel catfish (*Ictalurus punctatus*, 3·3–3·8 %)[6], flounder (*Paralichthys olivaceus*, 4·08 %)[7], orange-spotted grouper (*Epinephelus coioides*, 2·7 %)[8], hybrid catfish (*Clarias gariepinus* × *Clarias macrocephalus*, 4·45 %–5·0 %)[9], black sea bream (*Sparus macrocephalus*, 7·74 %)[10], yellow grouper (*Epinephelus awoara*, 6·5 %)[11], cobia (*Rachycentron canadum*, 5·57 %)[12], tilapia (*Oreochromis niloticus*, 6·24 %)[13] and blunt snout bream (*Megalobrama amblycephala*, 7·23 %)[14]. The studies mentioned above have shown that Arg requirements vary greatly among species. Thus, for formulating feeds of fish with no information about their Arg requirements, it is not feasible to use the value of dietary Arg requirement obtained from other fish species even if they are from the same family.

Abbreviations: AA, amino acid; Arg, arginine; IGF, insulin-like growth factor; Nrf2, NF-E2-related factor 2; PPV, protein productive value; TOR, target of rapamycin; WG%, weight gain %.

* **Corresponding author:** X. Wu, email wjurk@163.com

Dietary Arg can affect protein deposition and expression of growth-related genes in fish[15,16] and hence regulates fish growth, because protein deposition is closely related to fish growth response[17]. Protein deposition mainly depends on the balance between protein synthesis and degradation, and thus factors regulating protein synthesis or protein degradation can also affect protein deposition. For protein synthesis, one of its limiting steps is translation initiation regulated by the signalling pathway of target of rapamycin (TOR) in which eIF4E-binding protein (4E-BP) and ribosomal protein S6 kinase (S6k) are the important regulatory factors[18]. Previous studies have shown that dietary Arg supplementation stimulated the mTOR signalling pathway and protein synthesis in pig porcine trophectoderm cells[19] and rotavirus enteritis[20], and for fish similar results were also observed in Jian carp (*Cyprinus carpio* var. Jian)[21,22] and Gibel carp (*Carassis auratus gibelio*)[15].

Insulin-like growth factor (IGF), a major downstream target of growth hormone (GH), plays an important role in regulating growth and body size of animals[23,24]. It has been shown that Arg raised the release of GH and IGF-1 into the blood[25] and increased muscle gain[26] of animals. Arg also has an insulinotropic potential. In rainbow trout[27,28] and barfin flounder[29], it was observed that plasma insulin levels increased after Arg administration by intramuscular injection, intraperitoneal injection or dietary supplementation.

Besides the functions on fish growth performance, Arg also has an important role in the development of the gut and innate immune system of fish. Dietary Arg can improve protein digestion in the gastrointestinal tract[30] and stimulate intestinal cell migration and *ex vivo* intestinal protein synthesis[20,31]. The roles of Arg in the immune system have been shown in many animals[4,5]. Arg increases macrophage and natural killer cell cytotoxicity, increases the synthesis of IL-2 and CD3 expression in T cells[32,33] and modulates lymphocyte subsets and positively affects their adhesion molecules, chemotaxis and proliferation[33–35]; it also increases cell-mediated immunity and antibody titres in poultry after vaccination[36–38]. However, as with all nutrients there is, in each species an optimal intake of Arg and excessive Arg supplementations to diets can depress the growth performance and immune response in fish[5,14].

Hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) is one of the important intensively farmed marine fish species because of its high commercial value. The optimal protein and lipid requirements, protein:energy ratio and the reference dietary amino acid (AA) profile of hybrid grouper have been established in our previous studies[39–41]. However, until now, information on Arg nutrition in hybrid grouper is still unavailable. Thus, the aim of this study was to evaluate effects of dietary Arg levels on growth, intestine morphology, oxidation resistance and immunity of hybrid grouper juveniles, and to use this information to determine the optimal dietary Arg requirement of this fish species.

Methods

Experimental diets

Seven isoenergetic (1465 kJ (350 kcal)/100 g DM), isoproteic (53·5 % of DM) and isolipidic (7 % of DM) experimental diets were formulated to contain graded L-Arg levels ranging from 1·9 to 4·7 % (of DM) at approximately 0·5 % increments (Table 1).

Table 1. Formulations and analysed composition of experimental diets (DM basis)

	Dietary arginine levels (%)						
	1·9	2·2	2·7	3·1	3·8	4·1	4·7
Ingredients							
Peruvian fishmeal (anchovy)*	30	30	30	30	30	30	30
Casein	14	14	14	14	14	14	14
Chile fish oil (salmon)†	3·39	3·39	3·39	3·39	3·39	3·39	3·39
Vitamin mixture‡	1	1	1	1	1	1	1
Mineral mixture§	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Soyabean lecithin	1	1	1	1	1	1	1
Maize starch	16·07	16·07	16·07	16·07	16·07	16·07	16·07
Carboxymethyl cellulose	3	3	3	3	3	3	3
Cellulose	12·28	12·28	12·28	12·28	12·28	12·28	12·28
Amino acid mixture‖	15·76	15·76	15·76	15·76	15·76	15·76	15·76
L-Arg	0	0·5	1	1·5	2	2·5	3
Asp + Glu (1:1)	3	2·5	2	1·5	1	0·5	0
Analysed composition of diets¶							
DM (%)	85·9	86·4	87·7	88·9	88·3	88·4	88·7
Crude protein (%)	53·2	53·2	53·2	53·2	53·2	53·2	53·2
Crude lipid (%)	7·0	6·8	6·9	7·2	7·4	7·3	7·2
Arg	1·9	2·2	2·7	3·1	3·8	4·1	4·7

* Yongsheng Feed Corporation; proximate composition (% DM): moisture, 8·9; crude protein, 70·0; crude lipid, 8·7.
† High Fortune (Fujian) Bio-Tech Co. Ltd.
‡ Vitamin mixture (mg/g mixture): thiamine hydrochloride, 2·5; riboflavin, 10; calcium pantothenate, 25; nicotinic acid, 37·5; pyridoxine hydrochloride, 2·5; folic acid, 0·75; inositol, 100; ascorbic acid, 50; choline chloride, 250; menadione, 2; α-tocopheryl acetate, 20; retinol acetate, 1; cholecalciferol, 0·0025; biotin, 0·25; vitamin B_{12}, 0·05. All ingredients were diluted with α-cellulose to 1 g[42].
§ Mineral mixture (mg/g mixture): calcium lactate, 327; K_2PO_4, 239·8; $CaHPO_4.2H_2O$, 135·8; $MgSO_4.7H_2O$, 132; $Na_2HPO_4.2H_2O$, 87·2; NaCl, 43·5; ferric citrate, 29·7; $ZnSO_4.7H_2O$, 3; $CoCl_2.6H_2O$, 1; $MnSO_4.H_2O$, 0·8; KI, 0·15; $AlCl_3.6H_2O$, 0·15; $CuCl_2$, 0·1[42].
‖ Amino acid mixture (g/100 g): L-lysine, 1·57; L-methionine, 0·53; L-threonine, 0·89; L-isoleucine, 0·93; L-leucine, 1·64; L-phenylalanine, 0·53; L-valine, 0·70; L-histidine, 0·38; L-aspartic acid, 1·41; L-serine, 0·59; L-glutamic acid, 2·71; glycine, 1·61; L-alanine, 1·59; L-cystine, 0·35; L-tyrosine, 0·21; L-proline, 0·11.
¶ Values represent means of duplicate samples.

Table 2. Amino acid (AA) compositions (%) of experimental diets (DM basis)*

	Dietary arginine levels (%)						
AA/∑AA	1·9	2·2	2·7	3·1	3·8	4·1	4·7
Essential AA							
Lys	4·25	4·25	4·30	4·20	4·25	4·19	4·25
Arg	1·90	2·23	2·69	3·07	3·75	4·13	4·70
Met	1·39	1·41	1·46	1·43	1·47	1·39	1·44
Thr	2·43	2·43	2·45	2·41	2·45	2·39	2·42
Ile	2·51	2·49	2·52	2·47	2·51	2·50	2·55
Leu	4·72	4·70	4·75	4·72	4·76	4·69	4·73
Phe	2·12	2·14	2·15	2·11	2·14	2·08	2·14
Val	2·70	2·68	2·75	2·69	2·72	2·69	2·70
His	1·28	1·30	1·30	1·28	1·31	1·29	1·30
∑Essential AA	23·30	23·63	24·38	24·38	25·36	25·35	26·24
Non-essential AA							
Aspartic acid	6·02	5·83	5·64	5·30	5·13	4·77	4·59
Ser	2·17	2·15	2·18	2·12	2·15	2·08	2·13
Glutamic acid	10·23	10·00	9·83	9·40	9·25	8·78	8·66
Gly	3·16	3·23	3·22	3·18	3·27	3·18	3·21
Ala	3·42	3·48	3·51	3·47	3·53	3·48	3·52
Cys	0·29	0·33	0·32	0·38	0·39	0·43	0·46
Tyr	1·00	1·01	1·04	0·94	1·04	0·94	1·04
Pro	2·20	2·12	2·19	2·03	2·10	2·06	2·10
∑Non-essential AA	28·49	28·15	27·92	26·83	26·87	25·72	25·69
∑AA	51·79	51·77	52·30	51·22	52·23	51·08	51·93

* Values represent means of duplicate samples.

On the basis of the results from our previous studies[39–41], 53·5 % dietary crude protein level and 7 % dietary crude lipid level were selected, and the anchovy fishmeal AA profile was used as the reference for experimental diets. Gross energy levels of experimental diets were calculated using physiological fuel values described in published studies[43,44]. A mixture of crystalline AA was supplemented to simulate the fishmeal AA pattern of anchovy, leaving Arg out. A mixture containing equal proportions of aspartic acid and glutamate (1:1) was used to substitute for Arg in the low-Arg diets. These AA were used for the substitution because they are non-essential to groupers and are abundant in the anchovy AA profile. Targeted dietary Arg concentrations were 1·9, 2·2, 2·7, 3·1, 3·8, 4·1 and 4·7 % of DM, respectively (Table 2).

All dry ingredients were carefully weighed and mixed in a Hobart mixer (A-200T Mixer Bench Model unit; Resell Food Equipment Ltd) for 30 min, followed by gradual addition of the lipids during constant mixing.. Subsequently, 30–50 ml of water/100 g of DM was slowly blended into the premixed ingredients. The diets were produced in a noodle-like shape of 3 mm in diameter using a twin-screw meat grinder (Institute of Chemical Engineering, South China University of Technology) and then pelleted. All diets were air-dried at about 21°C for 24 h, sieved and then packaged and stored frozen (−20°C).

Experimental procedure

Hybrid grouper juveniles were obtained from a commercial hatchery (Wenchang, Hainan, China). Before the trial, experimental fish were acclimated with a commercial diet for 15 d, and then groups of sixteen fish (average initial body weight: 11·7 (SD 0·1) g) were randomly distributed into twenty-one 135-litre aquariums (length 60 cm × width 45 cm × height 50 cm) connected to mechanical and biological water filters as a recycling system. Each diet was randomly assigned to three replicate groups of fish. All aquaria received flowing sea water (salinity: 33·1 g/l) from the same reservoir at a rate of 3 litres/min. Throughout the trial, dissolved O_2 content in the fish tanks was measured every other day with a portable metre (HATCH HQ30d; Hatch Lange GMBH) and values ranged from 5·8 to 6·4 mg/l. Ammonia (0–0·19 mg/l) was measured with a portable spectrophotometer (HATCH DR 2800; Hatch Lange GMBH). Water temperature was daily registered using maximum–minimum thermometers and maintained at 27–28°C. Fish were exposed to a 12 h light–12 h dark cycle and fed each dietary treatment twice daily (08.00 and 16.00 hours) to apparent satiation. Feed intake was recorded daily and experimental aquaria were cleaned once a week. The growth trial was continued for 8 weeks.

Sampling and analysis

At the beginning of this trial, ten fish were sampled and stored at −20°C for analysis of initial whole-body proximate composition. At the end of the trial, the fish were euthanised with MS-222 (0·1 g/l), and two fish per aquarium were collected for whole-body composition analysis. For the biochemical analysis of serum, another three fish from each aquarium were separately bled from the caudal vasculature using 1-ml heparinised (H6279; SIGMA) syringes, and then liver and head kidney samples were collected and immediately frozen in liquid N_2 and then stored at −80°C. After centrifugation (3000 ***g***, 15 min, 4°C) (centrifuge 5417 R; Eppendorf), serum was separated and stored at −80°C until analysis. The muscle samples for compositional analysis and molecular analyses, as well as intestine samples for histological analysis, were also taken at this dissection.

Crude protein (N × 6·25) was determined by the Kjeldahl method after acid digestion using an auto Kjeldahl System (FOSS Tecator). Crude lipid was determined by diethyl ether extraction using a Soxtec System HT (Soxtec System HT6, SOX406; Haineng). DM was determined by heating approximately 2-g samples at 125°C for 3 h, and ash was quantified after heating approximately 2-g samples at 650°C for 3 h according to Association of Official Analytical Chemists[45]. The AA levels of the diets, muscle and serum were determined after acid hydrolysis using the L-8900 AA analyzer (Hitachi)[46].

Histological examination of the mid gut

For histological analysis, all gut specimens were washed in saline solution and fixed in 10 % neutral-buffered formalin for 24 h. After serial dehydration steps in alcohol, samples were embedded in paraffin. The blocks of embedded tissue were sectioned at 5 mm, and sections were routinely stained with haematoxylin–eosin and observed under a light microscope (Olympus). Average villus heights, enterocyte height, muscular layer thickness and serosal thickness were determined per slice by computer-operated image picture analysis system (Image-Pro Plus 7), with a digital camera attached to a light microscope. Six measurements of each parameter were made in each section (three fish per tank). In all, fifty-four measurements per treatment were used to calculate the average values.

Oxidative stress challenge test

After sampling, all remaining fish were fed their prescribed diets for 2 d and then exposed to 4·5 mg Cu(II)/l water for 36 h by the addition of $CuSO_4$ to water. The dose of Cu(II) exposure used in this study was found to induce oxidative stress in a preliminary experiment. During the challenge test, the water recirculation of the aquarium system was stopped by turning off the pump. The survival rate per aquarium was recorded, and then three fish per aquarium were randomly selected and individually sampled as above to obtain serum, liver and head kidney.

Total RNA extraction and reverse transcription

Total RNA was extracted from grouper liver and head kidney using Trizol Reagent (Invitrogen) followed by quality measurement on a 1·0 % denaturing agarose gel and yield determination on a NanoDrop® ND-1000. The RNA was treated with RNA-Free DNase (Takara) to remove DNA contamination and reversely transcribed to complementary DNA (cDNA) by the RevertAid First Stand cDNA Synthesis kit (Thermo Scientific) according to the instructions provided by the manufacturer.

Real-time quantitative PCR analysis of insulin-like growth factor-I, target of rapamycin, S6 kinase 1 and eIF4E-binding protein in liver, and NF-E2-related factor 2, Kelch-like-ECH associated protein 1, IL-1β, IL-8 and heat-shock protein 70 in head kidney

Real-time RT-PCR was carried out in a quantitative thermal cycler (Mastercyclereprealplex; Eppendorf). The amplification was performed in a total volume of 20 µl containing 10 µl of power SYBR® Green PCR Master Mix (Applied Biosystems), 1 µl of each primer (10 µmol/l), 6 µl of nuclease-free water and 2 µl of cDNA mix. The real-time RT-PCR programme was as follows: 95°C for 10 min, followed by forty cycles of 95°C for 15 s, 60°C for 60 s and 70°C for 20 s. The real-time RT-PCR primer pairs for *IGF-1, TOR*, S6 kinase 1 (*S6K1*), *4E-BP2*, NF-E2-related factor 2 (*Nrf2*), Kelch-like-ECH associated protein 1 (*Keap1*), *IL-1β*, *IL-8* and heat-shock protein 70 (*HSP70*) and *β-actin* were designed by Primer Premier 5.0 based on the published nucleotide sequences and listed in Table 3. At the end of each PCR reaction, melting curve analysis of amplification products was carried out to confirm that a single PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analysed according to the following equation $E = 10^{(-1/\text{slope})} - 1$. The expression levels of the target genes were calculated according to the $2^{-\Delta\Delta C_t}$ method described by Yao *et al.*[47].

Statistical analysis

Normality and homoscedasticity assumptions were confirmed before any statistical analysis. All evaluated variables were subjected to an ANOVA to determine whether dietary Arg levels significantly ($P < 0{\cdot}05$) affected the observed responses. In addition, to determine whether the effect was quadratic, a follow-up trend analysis using orthogonal polynomial contrasts was performed[48] using the SPSS 18.0 (SPSS Inc.). The adjusted R^2 (adjusted R^2) was calculated as previously described by Kvalseth[49]. The optimum dietary Arg requirement based on WG% or protein productive value (PPV) was established through the quadratic regression model.

Table 3. Primers used for quantitative RT-PCR (qPCR)

Used for	Gene name	Genbank accession no.	Primer sequence(5'–3')
qPCR	*TOR*	JN850959.1	F: TCTCCCTGTCCAGAGGCAATAA R: CAGTCAGCGGGTAGATCAAAGC
	S6K1	EF373684.1	F: GGTGCATGTCACCTTATGGG R: AGCTGGCAGCACTTCTAGTC
	4E-BP2	NM_001165149.1	F: GACCACTGCCAAGGCCATC R: CTGAACAGGGTTCCTCCGG
	IGF-1	AY776159.1	F: TATTTCAGTAAACCAACAGGCTATG R: TGAATGACTATGTCCAGGTAAAGG
	Keap1	XM_018665037.1	F: TCCACAAACCCACCAAAGTAA R: TCCACCAACAGCGTAGAAAAG
	Nrf2	KU892416.1	F: TATGGAGATGGGTCCTTTGGTG R: GCTTCTTTTCCTGCGTCTGTTG
	IL-1β	KP057877.1	F: CCTCATCATCGCCACACAGA R: TGCCTCACAACCGAACACAT
	IL-8	KC184490.1	F: AGTCATTGTCATCTCCATTGCG R: AAACTTCTTGGCCTGTCCTTTT
	TNF-α	AY667275.1	F: GAGGACGGTGGTGTTGGTGG R: TTCTCTTTGGCCTGATTGCG
	HSP70	AY423555.2	F: GTCCTGATCAAACGAAACACCA R: CACGCTCACCCTCATAAACCT
	β-Actin	AY510710.2	F: CTCTGGGCAACGGAACCTCT R: GTGCGTGACATCAAGGAGAAGC

TOR, target of rapamycin; F, forward sequence; R, reverse sequence; *S6K1*, ribosomal protein S6 kinase1; *4E-BP2*, eIF4E-binding protein; *IGF-1*, insulin-like growth factor-1; *Keap1*, Kelch-like-ECH associated protein1; *Nrf2*, nuclear factor erythroid 2-related factor 2-like 2; *HSP70*, heat-shock protein 70.

Results

Growth performance and feed utilisation

WG% of experimental fish was increased with the increasing dietary Arg levels, up to a peak value at 3·8 % dietary Arg level (Table 4), above which WG% was reduced. Quadratic regression analysis of WG% against dietary Arg levels indicated that optimal dietary Arg level was 3·65 % of DM (6·82 % of dietary protein) (Fig. 1) ($y = -38{\cdot}989x^2 + 284{\cdot}823x + 70{\cdot}077$, $R^2 = 0{\cdot}927$). Values of daily feed intake (DFI) of fish decreased as dietary Arg increased to 3·1 %, above which it increased with increasing rates of Arg inclusion. Feed conversion ratios (FCR) of fish fed 1·9, 2·2, 3·1 and 4·7 % dietary Arg levels were higher than those of fish fed 3·1, 3·8 and 4·1 % dietary Arg levels. Values of protein efficiency ratio (PER) and PPV were improved as dietary Arg level was increased from 1·9 to 3·1 %, and thereafter these values decreased as dietary Arg level continued to be increased. Quadratic regression analysis of PPV against dietary Arg levels indicated that optimal dietary Arg level was 3·55 % of DM (6·64 % of dietary protein) ($y = -2{\cdot}074x^2 + 14{\cdot}721x + 14{\cdot}412$, $R^2 = 0{\cdot}760$).

Table 4. Growth performance and feed utilisation of hybrid grouper juveniles fed different dietary arginine (Arg) levels for 8 weeks

Dietary Arg levels (%)	WG*	DFI†	FCR‡	PER§	PPV‖
1·9	472	1·62	0·93	2·03	34·54
2·2	509	1·60	0·90	2·10	37·00
2·7	552	1·52	0·87	2·21	38·97
3·1	574	1·47	0·85	2·29	40·92
3·8	591	1·49	0·85	2·25	40·12
4·1	590	1·52	0·85	2·22	39·02
4·7	543	1·56	0·87	2·15	38·30
PSE	11	0·03	0·02	0·05	0·92
ANOVA (*P*)	<0·001	0·001	0·001	0·002	<0·001
Regression analysis of SOP (*n* 3)					
Adj. R^2	0·919	0·687	0·730	0·675	0·733
P	<0·001	<0·001	<0·001	<0·001	<0·001

WG, weight gain; DFI, daily feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; PPV, protein productive value; PSE, pooled standard error of treatment means (*n* 3); SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.
* WG: 100 × (final mean body weight – initial mean body weight) / initial mean body weight.
† DFI = 100 × feed offered /average total weight/d.
‡ FCR: g dry feed/g weight gain.
§ PER: g weight gain/g protein fed.
‖ PPV: g protein gain/g protein fed.

Whole-body and muscle compositions

The protein contents of whole body and muscle were significantly influenced by the experimental treatments ($P < 0{\cdot}05$) (Table 5). Fish fed 3·8 % dietary Arg had higher whole-body and muscle protein contents than fish fed other levels of dietary Arg. Fish fed 1·9 % dietary Arg had the lowest whole-body and muscle protein contents among all experimental treatments.

Table 5. Whole-body and muscle compositions (fresh-weight basis) of hybrid grouper juveniles fed different dietary arginine (Arg) levels for 8 weeks

	Whole-body composition (*n* 6)			Dorsal muscle composition (*n* 9)		
Dietary Arg levels (%)	Moisture	Protein	Lipid	Moisture	Protein	Lipid
1·9	72·79	16·83	5·58	77·04	20·14	0·83
2·2	71·88	17·19	5·66	76·72	20·21	0·85
2·7	71·63	17·39	6·11	76·90	20·19	0·87
3·1	72·49	17·47	5·52	76·35	20·83	0·90
3·8	72·42	17·94	5·08	76·31	20·85	0·85
4·1	72·86	17·38	4·97	76·37	20·83	0·87
4·7	72·58	17·55	4·81	76·97	20·53	0·81
PSE	0·66	0·23	0·36	0·40	0·09	0·06
ANOVA (*P*)	0·486	0·010	0·032	0·337	<0·001	0·876
Regression analysis of SOP						
Adj. R^2	−0·030	0·441	0·384	0·153	0·655	0·025
P	0·507	0·002	0·005	0·087	<0·001	0·309

PSE, pooled standard error of treatment means (*n* 3); SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

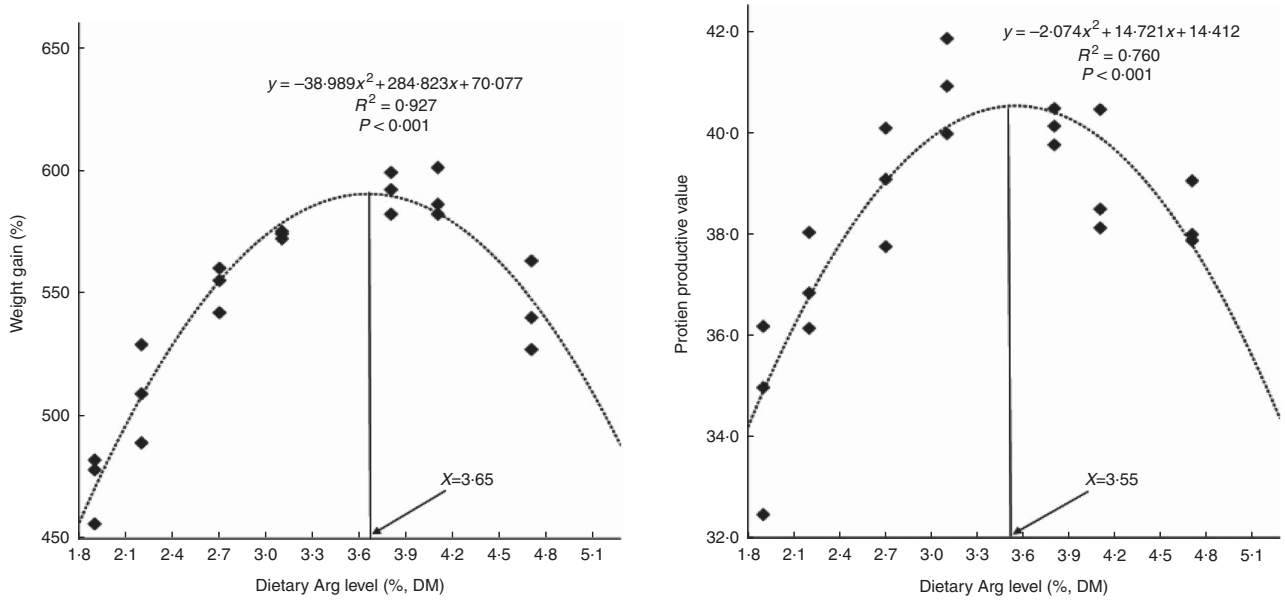

Fig. 1. Relationship of weight gain % and protein productive value with dietary arginine (Arg) levels of hybrid grouper juveniles.

Whole-body lipid contents of fish fed 1·9, 2·2, 2·7 and 3·1 % were higher than those of fish fed 3·8, 4·1 and 4·7 %. There were no significant differences in whole-body moisture, muscle lipid and muscle moisture among any of the experimental treatments.

Amino acid concentrations in muscle and serum

Contents of individual AA, such as lysine, methionine, threonine, valine, aspartic acid, glutamic acid, alanine, the total essential amino acids and non-essential amino acids in muscle of fish fed 3·1 and 3·8 % dietary Arg levels were higher than those in muscle of fish fed 1·9, 2·2 and 2·7 % dietary Arg levels (Table 6). Fish fed 3·8 % dietary Arg level had higher Arg concentrations in muscle compared with fish fed other experimental diets. Muscle histidine contents of fish fed 3·1, 3·8 and 4·1 % dietary Arg levels were higher than those of fish fed 1·9, 2·2, 2·7 and 4·7 % dietary Arg levels. Muscle proline contents of fish fed 2·2, 2·7 and 4·7 % dietary Arg levels were lower than those of fish fed other experimental diets.

In serum, fish fed 1·9, 2·2 and 2·7 % dietary Arg levels showed significantly lower Arg concentrations compared with fish fed 3·1, 3·8, 4·1 and 4·7 % dietary Arg levels (Table 7). Fish fed 1·9, 2·2, 4·1 and 4·7 % dietary Arg levels had lower serum glutamic acid concentrations compared with fish fed 2·7, 3·1 and 3·8 % dietary Arg levels. Fish fed 3·8 % dietary Arg level exhibited the highest serum glutamine content among all experimental treatments. Citrulline and ornithine contents in serum were too low to be detected by the methods used.

Gut morphometric analysis

Morphometric measurements of mid gut are summarised in Table 8, and histological examination images are shown in Fig. 2. Villus height, enterocyte height, muscular layer thickness and serosa thickness of mid gut showed obvious responses to dietary Arg supplementations. Villus height of fish fed 1·9 and

Table 7. Serum amino acids (mg/100 ml) of juvenile hybrid grouper fed different dietary arginine (Arg) levels for 8 weeks

Dietary Arg levels (%)	Arg	Glutamic acid	Glx	Cit	Orn
1·9	13·33	17·77	17·57	–	–
2·2	13·50	17·50	18·73	–	–
2·7	11·47	21·07	16·43	–	–
3·1	20·17	21·73	17·93	–	–
3·8	22·10	20·40	22·17	–	–
4·1	19·23	17·57	19·63	–	–
4·7	20·23	17·30	17·20	–	–
PSE	0·62	0·57	0·43		
ANOVA (*P*)	<0·001	<0·001	<0·001		
Regression analysis of SOP (*n* 9)					
Adj. R^2	0·603	0·586	0·099		
P	<0·001	<0·001	0·152		

–, not detected; PSE, pooled standard error of treatment means (*n* 3); SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

Table 8. Gut morphology (μm) of juvenile hybrid grouper fed different dietary arginine (Arg) levels for 8 weeks

Dietary arginine levels (%)	Villus height	Enterocyte height	Muscular layer thickness	Serosa thickness
1·9	285·33	10·87	32·00	12·67
2·2	382·67	14·20	38·67	21·33
2·7	558·67	13·77	47·67	26·67
3·1	581·33	18·00	54·00	26·00
3·8	661·00	25·67	65·67	42·00
4·1	540·67	18·10	74·00	46·00
4·7	633·67	20·33	59·67	31·67
PSE	37·91	2·26	5·20	2·44
ANOVA (*P*)	<0·001	<0·001	<0·001	<0·001
Regression analysis of SOP (*n* 9)				
Adj. R^2	0·783	0·513	0·777	0·745
P	<0·001	0·001	<0·001	<0·001

PSE, pooled standard error of treatment means (*n* 3); SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

Table 6. Dorsal muscle amino acids (g/100 g wet weight) of juvenile hybrid grouper fed different dietary Arg levels for 8 weeks

	Dietary arginine levels (%)							ANOVA		SOP regression (*n* 9)	
	1·9	2·2	2·7	3·1	3·8	4·1	4·7	PSE	*P*	Adj. R^2	*P*
Essential amino acids											
Lys	1·85	1·86	1·86	1·94	1·92	1·90	1·87	0·01	<0·01	0·506	0·001
Arg	1·22	1·23	1·23	1·25	1·27	1·26	1·23	0·01	0·007	0·374	0·006
Met	0·62	0·62	0·63	0·66	0·64	0·63	0·62	0·01	0·004	0·465	0·001
Thr	0·97	0·96	0·96	1·01	1·01	0·99	0·97	0·01	<0·001	0·307	0·014
Ile	0·96	0·97	0·97	1·01	0·99	0·98	0·97	0·01	0·062	0·227	0·038
Leu	1·68	1·68	1·67	1·73	1·73	1·71	1·70	0·02	0·063	0·203	0·051
Phe	0·84	0·81	0·82	0·77	0·78	0·82	0·83	0·04	0·596	0·045	0·257
Val	0·97	0·99	1·00	1·05	1·03	1·00	0·99	0·02	0·003	0·425	0·003
His	0·44	0·44	0·44	0·46	0·46	0·46	0·44	0·01	0·034	0·360	0·007
∑Essential amino acids	9·55	9·55	9·56	9·85	9·85	9·74	9·62	0·08	0·002	0·422	0·003
Non-essential amino acids											
Aspartic acid	2·16	2·14	2·15	2·22	2·21	2·18	2·16	0·02	0·004	0·252	0·028
Ser	0·79	0·80	0·80	0·83	0·82	0·81	0·79	0·01	0·007	0·398	0·004
Glutamic acid	3·12	3·11	3·10	3·23	3·21	3·17	3·15	0·03	0·001	0·302	0·015
Gly	1·09	1·12	1·11	1·16	1·14	1·13	1·12	0·02	0·104	0·248	0·030
Ala	1·27	1·27	1·25	1·32	1·30	1·28	1·26	0·01	<0·001	0·195	0·055
Tyr	0·67	0·67	0·66	0·67	0·69	0·68	0·69	0·02	0·522	0·079	0·185
Pro	0·67	0·64	0·63	0·69	0·69	0·70	0·65	0·02	0·007	0·028	0·299
∑Non-essential amino acids	9·78	9·77	9·71	10·11	10·07	9·97	9·83	0·07	<0·001	0·330	0·011

SOP, second-order polynomial trend; PSE, pooled standard error of treatment means (*n* 3); Adj. R^2, adjusted R^2.

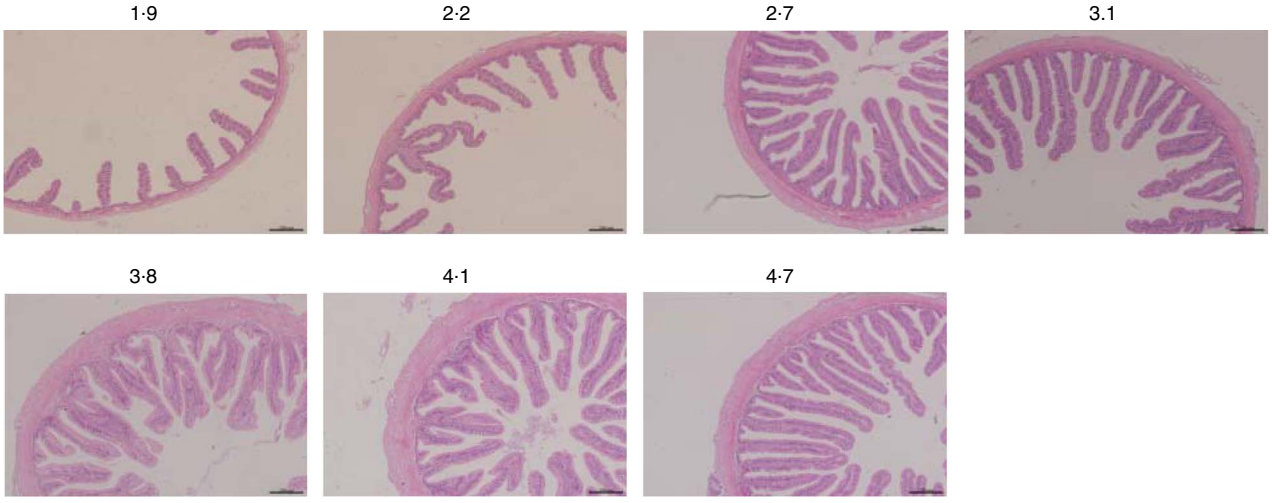

Fig. 2. Light microscopy of the mid gut morphology of hybrid grouper juveniles in fish fed different dietary arginine levels for 8 weeks (haematoxylin–eosin staining; original magnification 10×).

2·2 % dietary Arg levels were significantly lower than that of fish fed other experimental diets. Fish fed 1·9, 2·2 and 2·7 % dietary Arg levels had lower enterocyte height compared with fish fed 3·1, 3·8, 4·1 and 4·7 % dietary Arg levels. Muscular layer thickness of mid gut was improved as dietary Arg level increased from 1·9 to 4·1 %, and thereafter it was decreased as dietary Arg level increased from 4·1 to 4·7 %. Serosa thickness of mid gut of fish showed a similar tendency as muscular layer thickness, and fish fed 3·8 and 4·1 % dietary Arg levels had significantly higher serosa thickness compared with fish fed other dietary Arg levels.

The relative mRNA expression levels of hepatic insulin-like growth factor-I, target of rapamycin, S6 kinase 1 and eIF4E-binding protein genes

The relative mRNA expression levels of the hepatic *IGF-1* gene in fish fed 3·8 and 4·1 % dietary Arg levels were higher than those in fish fed 1·9, 2·2, 2·7, 3·1 and 4·7 % dietary Arg levels (Fig. 3). Fish fed 1·9, 2·1 and 4·7 % dietary Arg levels had lower hepatic mRNA levels of the *TOR* gene compared with fish fed 2·7, 3·1, 3·8 and 4·1 % dietary Arg levels. Fish fed 3·8 % dietary Arg level had the highest relative mRNA expression levels the *TOR* gene in liver among all experimental treatments. The relative hepatic mRNA expression levels of the *S6K1* gene increased as dietary Arg level increased, reaching a peak value at 3·8 % dietary Arg level, and then they decreased when dietary Arg level continued to rise to 4·1 or 4·8 %. There were no significant differences in the relative mRNA expression levels of the *4E-BP2* gene in liver among fish fed different dietary Arg levels.

Survival, serum immune indices and hepatic antioxidant indices

Before challenge (exposure to 4·5 mg Cu(II)/l water for 36 h). Fish survival ratios and serum lysozyme activity showed no remarkable variations among all experimental treatments after they were fed experimental diets for 8 weeks (Fig. 4). Fish fed 3·8 % dietary Arg level had significantly higher serum IgM concentrations than fish fed 1·9, 2·2, 2·7 or 4·7 % dietary Arg level. Fish fed 1·9 % dietary Arg level displayed lower serum IgM concentrations than fish fed other dietary Arg levels. Fish fed 3·1 and 3·8 % dietary Arg levels had higher hepatic catalase (CAT) activities compared with fish fed other levels of dietary Arg (Fig. 5). Fish fed 3·8 % or 4·1 % dietary Arg level had lower malondialdehyde (MDA) contents in liver compared with fish fed other dietary Arg levels, and fish fed 1·9 % dietary Arg level had the highest MDA content in liver among all experimental treatments.

After challenge (exposure to 4·5 mg Cu(II)/l water for 36 h). Fish fed 1·9 and 2·2 % Arg levels had lower survival ratios than fish fed higher (2·7–4·7 % dietary) Arg levels after exposure to 4·5 mg Cu^{2+}/l water for 36 h. Serum lysozyme activities in fish fed 1·9, 2·2 and 2·7 % dietary Arg levels were lower than those in fish fed higher dietary Arg levels. Fish fed 3·8 % dietary Arg level had higher serum IgM concentrations than fish fed other experimental diets, and serum IgM concentrations of fish fed 1·9 % dietary Arg level were the lowest among all experimental groups. Fish fed 1·9 % dietary Arg level had a lower value of CAT activity than fish fed 3·1 % dietary Arg level. Hepatic MDA contents in fish fed 1·9, 2·2, 2·7 and 3·1 % dietary Arg levels were higher than those in fish fed dietary Arg levels above these inclusion levels.

Expressions of immune genes in head kidney

Before copper-induced oxidative stress (exposure to 4·5 mg Cu(II)/l water for 36 h). There were no significant differences in *Nrf2* gene expressions of head kidney among all experimental groups (Fig. 6). Gene expression of the *KEAP1* gene in head kidney of fish fed 1·9, 2·2, 2·7 and 4·7 % dietary Arg levels were higher than those in fish given feed containing 3·1, 3·8 or 4·1 % Arg. Fish fed 1·9, 2·2, 2·7 and 4·7 % dietary Arg levels showed significantly lower gene expression of *IL-1β* in head kidney compared with fish fed 3·8 and 4·1 % dietary Arg levels. Transcript levels in head kidney for *IL-8* and *HSP70* showed no significant differences among groups.

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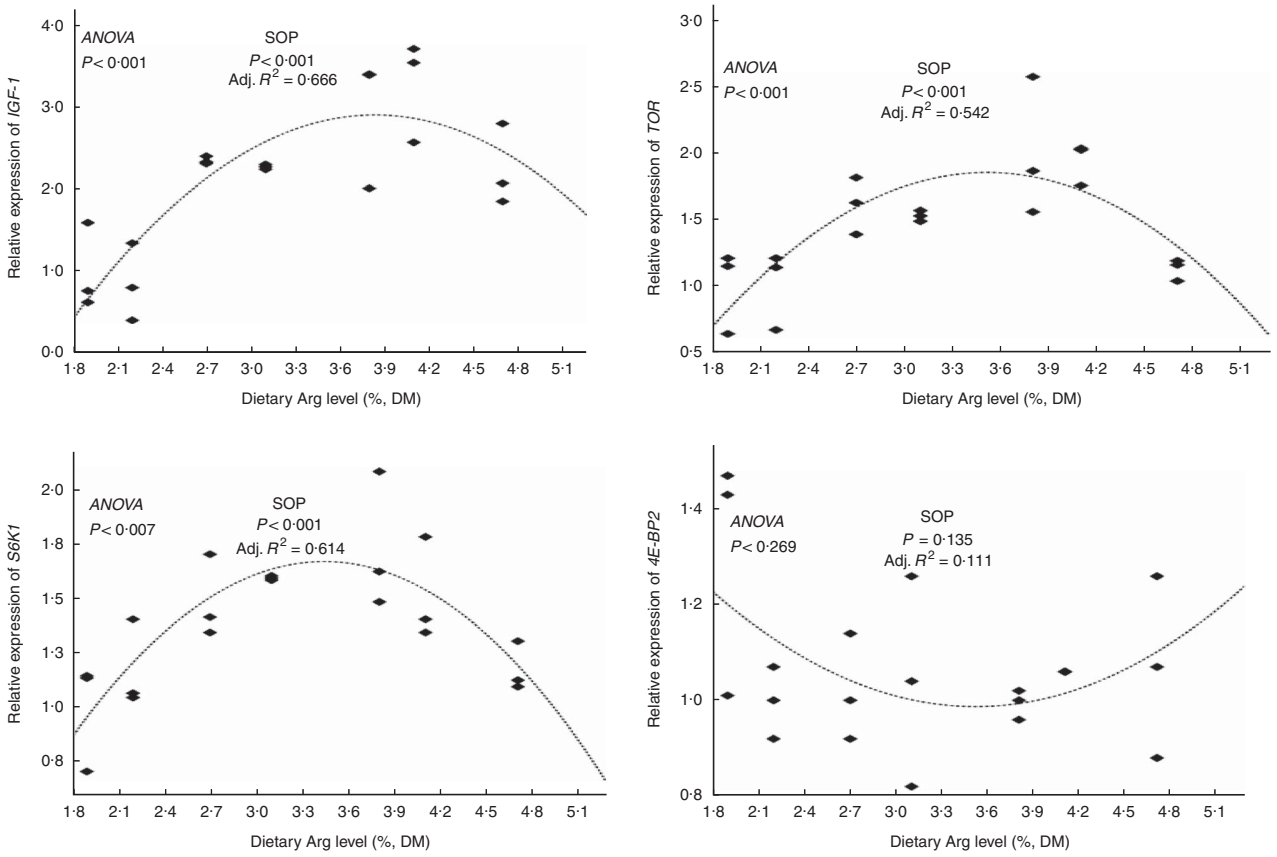

Fig. 3. Relative expression of hepatic insulin-like growth factor-1 (*IGF-1*), target of rapamycin (*TOR*), S6 kinase 1 (*S6K1*) and eIF4E-binding protein (*4E-BP2*) genes of hybrid grouper juveniles fed diets with different arginine (Arg) levels for 8 weeks (*n* 9). Relative mRNA expression was evaluated by real-time quantitative PCR. SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

After copper-induced oxidative stress (exposure to 4·5 mg Cu(II)/l water for 36 h)

Levels of *Nrf2* mRNA in head kidney of fish fed 1·9 2·2, 2·7 and 3·1 % dietary Arg were lower than those in fish fed other dietary Arg levels. Gene expression levels of *KEAP1* in head kidney of fish fed 1·9, 2·2 and 2·7 % dietary Arg levels were significantly higher than those in fish fed other dietary Arg levels. Fish fed 1·9, 2·2, 2·7 and 3·1 % dietary Arg levels had higher gene expression of *IL-1β* in head kidney compared with fish fed other dietary Arg levels. Expression levels of *IL-8* mRNA in head kidney of fish fed 1·9, 2·2 and 2·7 % dietary Arg levels were higher than those in fish fed other dietary Arg levels. Fish fed 1·9 and 2·2 % dietary Arg levels had lower expression of *HSP70* mRNA in head kidney compared with fish fed other dietary Arg levels.

Discussion

This study indicated that optimal dietary Arg requirement of hybrid grouper for maximum growth or PPV was estimated to be 3·55–3·65 % of DM, corresponding to 6·64–6·82 % of dietary protein. The optimal Arg requirement (% dietary protein) obtained in this experiment was close to that observed in tilapia (6·24 %)[13], cobia (6·20 %)[12], yellow grouper (6·5 %)[11] or silver perch (6·8 %)[50], but differed from that observed in catla (5·57 %)[51], rohu (3·05 %–3·47 %)[52], hybrid catfish (4·45 %–5·0 %)[9], flounder (4·08 %)[7], channel catfish (3·3 %–3·8 %)[6] and coho salmon (4·9 %)[53] or blunt snout bream (7·23 %)[14]. Requirements of dietary Arg obtained in different studies for fish species ranged from 3·0 to 8·1 % of dietary protein[1]. These differences might be attributed to the differences in fish species, experimental designs or analytical methods. In this study, both excess and insufficiency of dietary Arg resulted in the depression of fish growth. The poor growth of fish fed low dietary Arg levels was ascribed to the reduced growth rate, the low feed efficiency and protein retention, agreeing with the results reported in other fish species[54,55]. Similarly, the decreased WG of fish fed dietary Arg levels above the optimal requirement were found also in other fish species[9–11,21,27,55,56], and the reasons may be the low expression of *IGF-1* and *TOR* genes as observed in the present study. The mechanism of depressing fish growth by excessive intake of Arg needs to be further studied.

Arg can serve as a potent stimulant of insulin and GH[57]. It has been reported that dietary Arg supplementation affected IGF-1 secretion and hence altered fish growth[27,58]. In this study, it was also observed that dietary Arg levels affected hepatic *IGF-1* gene expressions. Fish fed 3·8 and 4·1 % dietary Arg levels had higher relative mRNA expression levels of hepatic *IGF-1* and better growth than fish fed lower or higher dietary Arg levels, which indicated a positive relationship between growth performance and hepatic *IGF-1* expression.

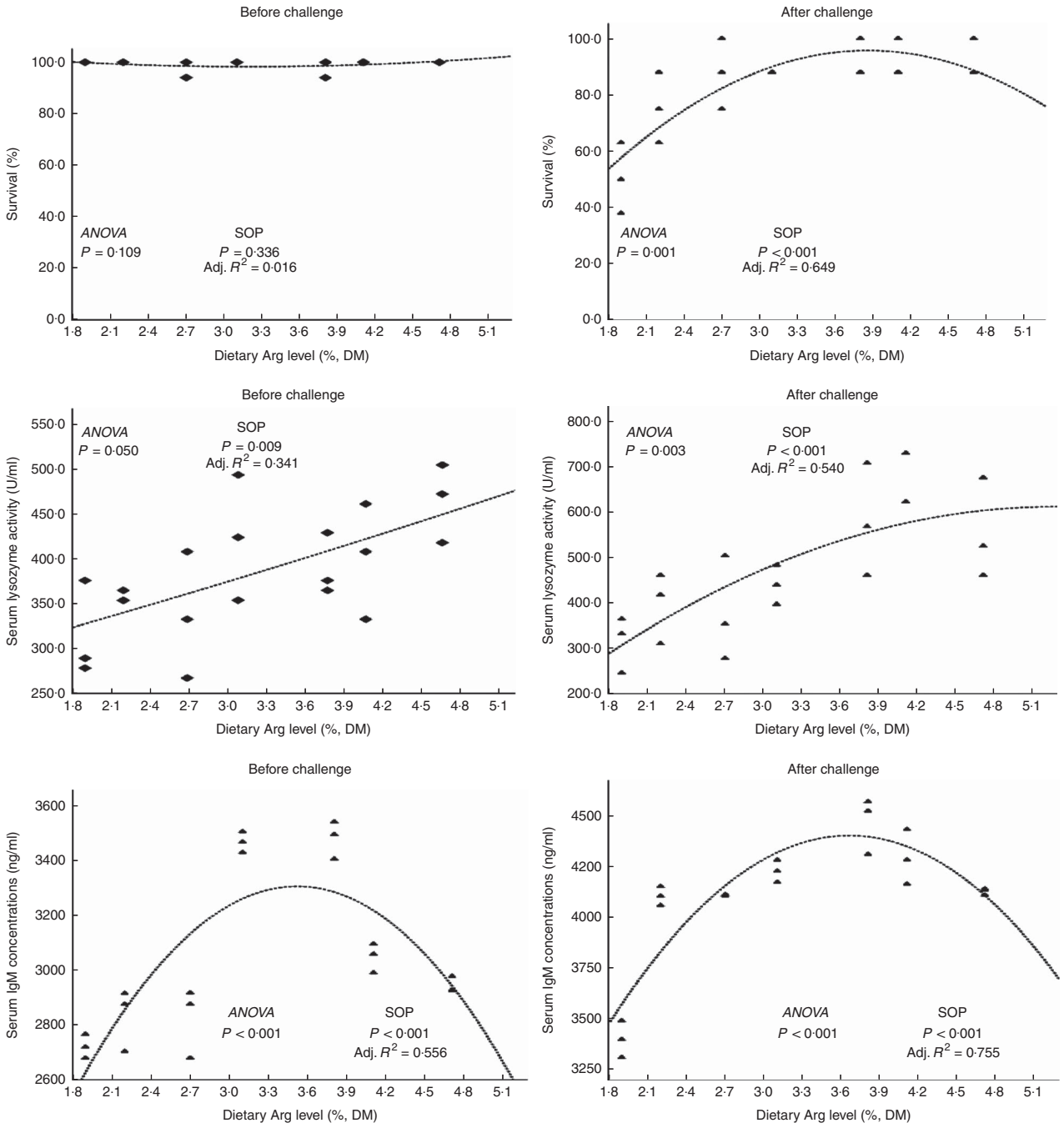

Fig. 4. Survival and serum lysozyme activity and IgM concentrations of hybrid grouper juveniles fed different dietary arginine (Arg) levels for 8 weeks before/after exposure to 4·5 mg Cu(II)/l water for 36 h (*n* 9). SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

The higher DFI observed in fish fed 1·9 and 2·2 % dietary Arg levels compared with those in fish fed other dietary Arg levels was attributed to their higher FCR, lower PER and PPV, which in turn resulted from Arg insufficiency. It has been shown that when fish are fed an imbalanced AA diet, the absorbed dietary AA are not matching the profile needed for protein synthesis, and become deaminated for use in energy production, gluconeogenesis or lipogenesis[59], accordingly leading to an increment in AA oxidation and catabolism[60,61], thus reducing PER and PPV.

The guts of vertebrates are quite sensitive to dietary nutritional alterations either in quality or in quantity[62]. Studies on gut morphology of humans and other terrestrial vertebrates have shown that dietary Arg affected gut health, alleviated gut mucosal injury and increased villus height[63–65]. In this study, gut morphology of fish, including the villus height, enterocyte height, muscular layer thickness and serosa thickness, generally were improved as dietary Arg level increased. Similar results were also observed in red drum[2] and hybrid striped bass[3].

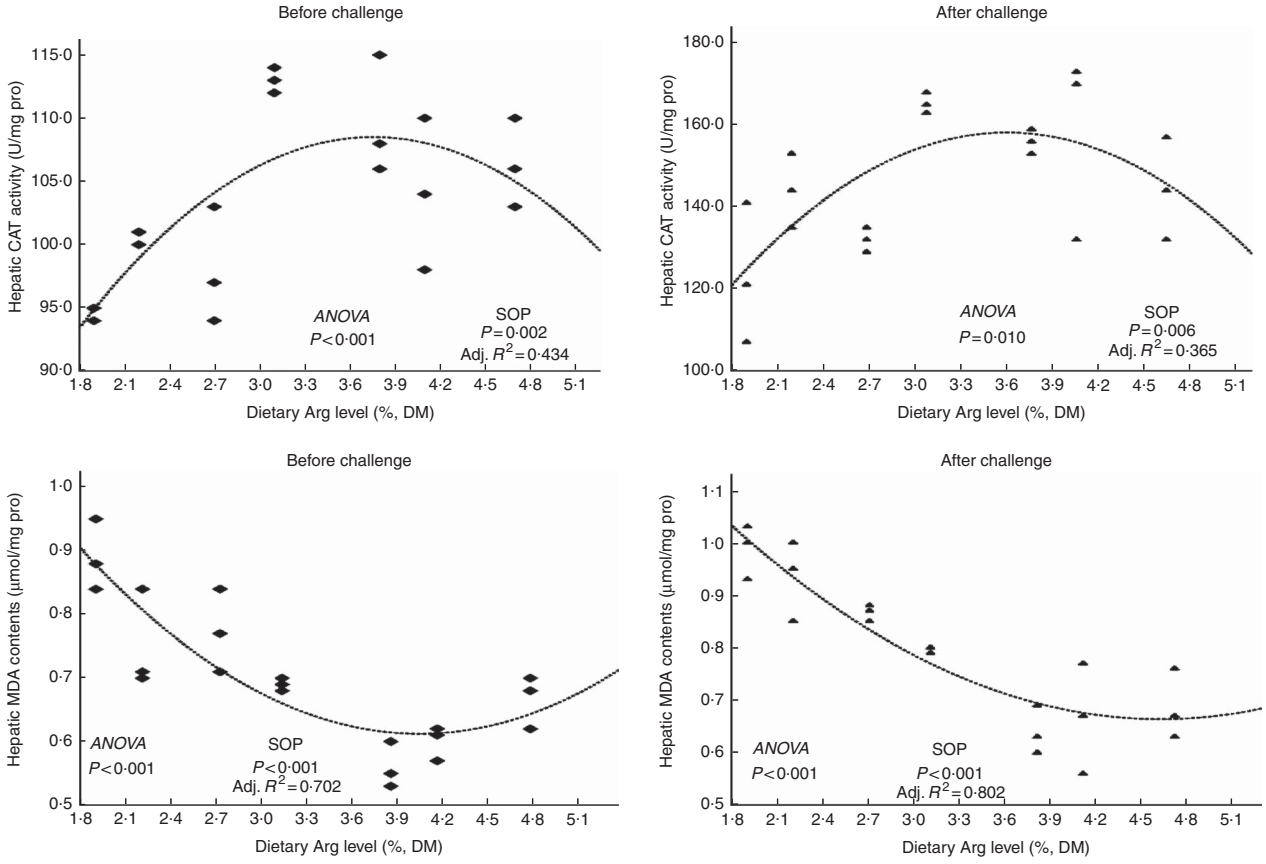

Fig. 5. Hepatic catalase (CAT) activity and malondialdehyde (MDA) contents of hybrid grouper juveniles fed different dietary arginine (Arg) levels for 8 weeks before/after exposure to 4·5 mg Cu(II)/l water for 36 h (*n* 9). SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

The higher muscle protein contents in fish fed 3·1, 3·8 and 4·1 % dietary Arg levels, compared with those in fish fed 1·9, 2·2 and 2·7 % dietary Arg, indicated that dietary Arg supplementation improved the N retention and protein deposition of hybrid grouper, which coincided with the higher PPV and muscular total AA contents observed in fish fed 3·1, 3·8 and 4·1 % dietary Arg levels. These results also agreed with data from the study of Liang *et al.*[16]. Protein deposition was obviously associated with AA metabolism in fish[66]. The role of Arg in protein synthesis was mainly through activation of the mTOR signalling pathway[16]. In mammals, dietary Arg supplementation can stimulate mTOR signalling activity[18,19,67] and protein synthesis[19]. In this study, suitable Arg supplementation levels in diets improved the transcription abundances of hepatic *TOR* and *S6K1* genes involved in protein synthesis, which is in accordance with the reports in juvenile gibel carp and blunt snout bream[15,16].

The contents of certain AA in fish muscle, blood or other tissue represent dietary AA status in fish nutrition studies[68–70]. Buentello & Gatlin[6] reported that dietary glutamate can be used as substrate for the endogenous synthesis of Arg in channel catfish, especially when dietary Arg is deficient. In this study, serum glutamate and glutamine contents did not display an obvious correlation with dietary glutamate levels, and circulating serum citrulline and ornithine were not detected. This indicated that exogenous dietary glutamate did not change into Arg in hybrid grouper at the present experimental conditions. The discrepancy in results might be explained by the differences in fish species, Arg and/or glutamate levels in diets, but further studies would be needed to clarify the underlying reason.

The variations in hepatic CAT activity and MDA content of experimental fish indicated that suitable inclusions of Arg to diets could improve hepatic antioxidant activity of hybrid grouper, but excess or insufficiency of dietary Arg did not have a positive role on fish resistance to oxidative stress, as induced by Cu exposure. Similar results were also observed in juvenile yellow catfish[5] and young grass carp[71,72]. The functional role of Arg in the antioxidant system of fish is still not well understood. However, L-Arg is the substrate of iNOS, and in rats L-Arg supplementation activated inducible NO synthase, superoxide dismutase, increased CAT2, NO and anti-O_2^{-1} levels in tissues and serum[73,74]. Wallner *et al.*[75] reported that the effect of L-Arg on the antioxidant defence is also associated with its anti-atherosclerotic effect.

Besides the influence on growth performance, antioxidant system and gut morphology observed in this study, dietary Arg levels were also found to significantly affect the innate immune responses of hybrid grouper regarding the alterations in serum lysozyme activity, IgM concentrations and expression of inflammation-related genes in head kidney. In this study, it was observed that before Cu challenge only serum IgM and *Keap1* and *IL-1β* mRNA in head kidney robustly responded to different

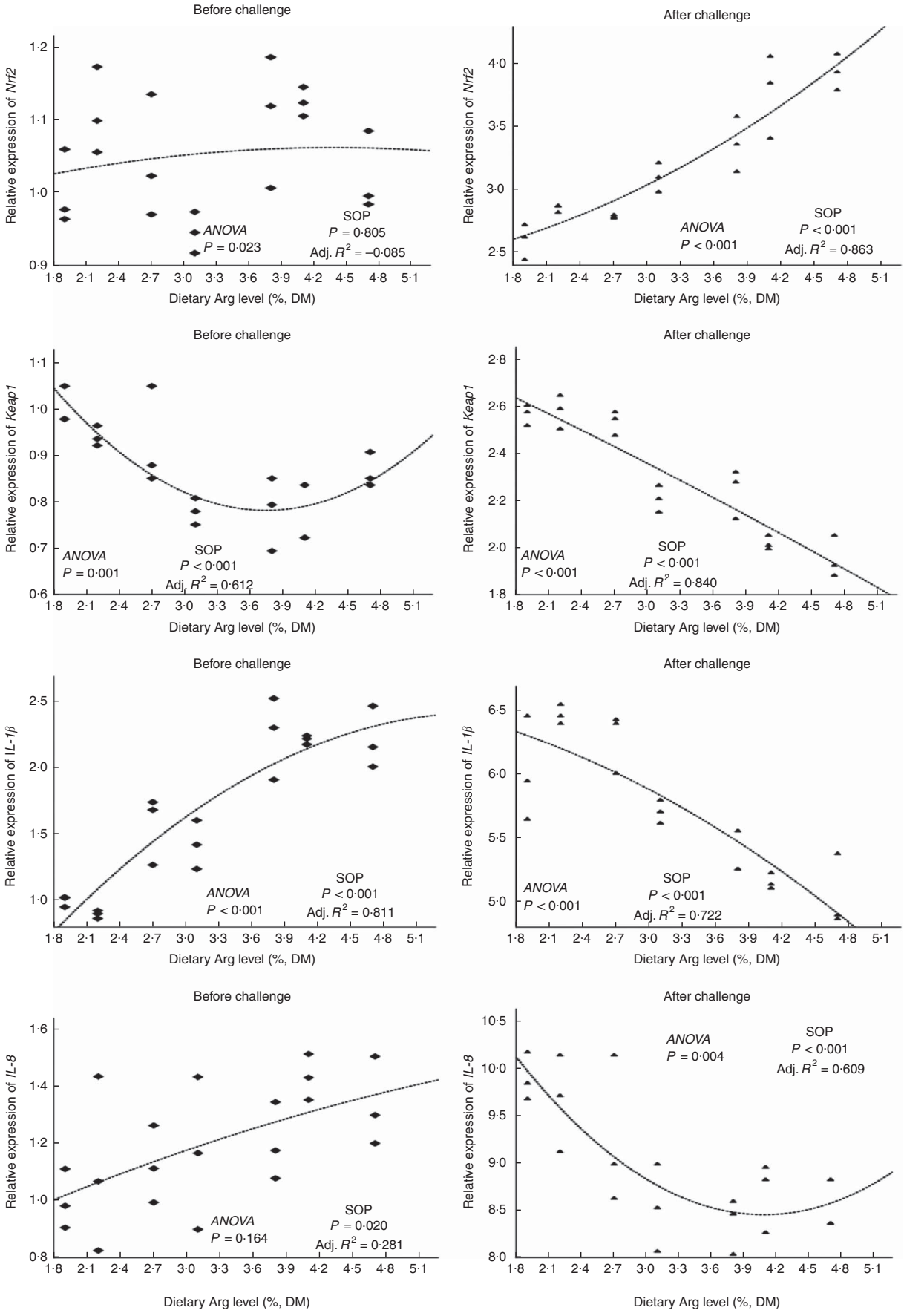

Fig. 6. *(Continued on following page)*

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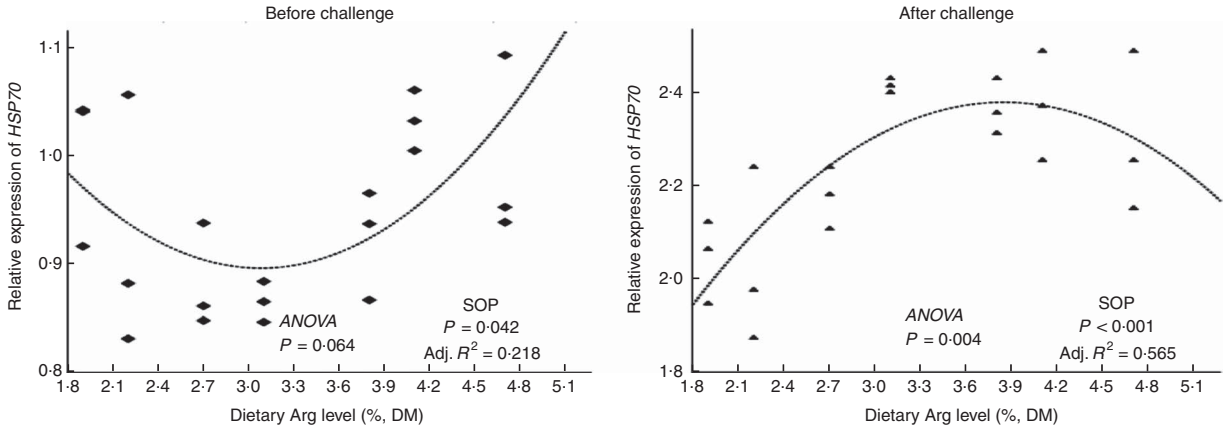

Fig. 6. *(Continued from previous page)* Relative expression of NF-E2-related factor 2 (*Nrf2*); Kelch-like-ECH-associated protein 1 (*Keap1*); *IL-1β*; *IL-8*; and heat-shock protein 70 (*HSP70*) genes in the head kidney of hybrid grouper juveniles fed diets with different Arg concentrations for 8 weeks before/after exposure to 4·5 mg Cu(II)/l water for 36 h (*n* 9). Relative mRNA expression was evaluated by real-time quantitative PCR. SOP, second-order polynomial trend; Adj. R^2, adjusted R^2.

dietary Arg levels, but after Cu challenge all immune parameters measured showed significant responses to different dietary Arg levels. These indicated that the activation of the immune system by suitable Arg supplementation in hybrid grouper mainly occurred when the fish was subjected to Cu stress. The role of Arg in improving innate immunity has been demonstrated in many fish species, such as red drum[2], hybrid striped bass[3], yellow catfish[5], golden pompano[76], Senegalese sole[77] and Jian carp[78]. Results from this study further showed that the mode of action by which Arg acts on the hybrid grouper's innate immunity is possibly by increasing serum lysozyme activity and IgM concentrations, together with stimulating the Nrf2-Keap1 signalling pathway and *HSP70* expression in head kidney, agreeing with the published studies above. Chen *et al.*[78] also reported that Arg supplementation could increase serum C3 and C4 levels of fish. However, although suitable Arg supplementation increased *Nrf2* and *HSP70* gene expression in this study, expression of two important proinflammatory mediators, *IL-1β* and *IL-8*, was also suppressed after the challenge, which might suggest that the immune response could be partly impaired. The higher cumulative mortalities of fish fed 1·9 and 2·2 % dietary Arg levels in comparison with those of fish fed other dietary Arg levels were attributed to their impaired gut development, oxidative stress resistance and innate immunity.

In conclusion, results of this study indicated that the optimal dietary Arg requirement for maximum growth or PPV of juvenile hybrid grouper was estimated to be 3·55–3·65 % of DM, corresponding to 6·64–6·82 % of dietary protein, which would be useful in developing AA balanced diets for juvenile hybrid grouper. Optimal dietary Arg supplementation improved gut morphology and resistance against oxidative stress in hybrid grouper.

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British Journal of Nutrition

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