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Effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*)

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ABSTRACT

The study was conducted to investigate the effects of dietary docosahexaenoic to eicosapentaenoic acid ratio (DHA/EPA) on growth, nonspecific immunity, immune related gene expression and disease resistance of juvenile large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Five isoproteic and isolipidic diets were formulated with graded ratios of DHA/EPA (0.61, 1.54, 2.17, 3.04 and 3.88) and the total amount of n−3 highly unsaturated fatty acids (n−3 HUFA) was approximately fixed at 1.0% of the dry weight. Each diet was randomly allocated to triplicate groups of fish in floating sea cages (1.0×1.0×1.5 m), and each cage was stocked with 60 fish (initial average weight 9.8±0.6 g). Fish were fed twice daily (05:00 and 17:00) to apparent satiation for 58 days. Results showed that specific growth rate (SGR) significantly increased from 2.03% d^{-1} to 2.26% d^{-1} ($P<0.05$) and then decreased with no significant differences ($P>0.05$). Nitro blue tetrazolium (NBT) positive leucocytes percentage of head kidney and serum lysozyme activity were significantly higher in fish fed diets with moderate (2.17) or higher DHA/EPA (3.14) ($P<0.05$). Hepatic Toll-like receptor 22 (TLR22) and Myeloid differentiation factor 88 (MyD88) expression levels were significantly increased in fish fed higher DHA/EPA especially at the early stage after natural infestation of parasites. In kidney, the expression of TLR22 was significantly up-regulated in fish fed moderate dietary DHA/EPA only at the early stage after natural infestation of parasites. The 13 day cumulative mortality rate following natural infestation of parasites decreased significantly with DHA/EPA increased from 0.61 to 3.04 ($P<0.05$), and then increased with DHA/EPA from 3.04 to 3.88 ($P>0.05$). Results of this study suggested that fish fed moderate or higher DHA/EPA had higher growth, nonspecific immunity immune related gene expression and disease resistance following natural infestation of parasites and dietary DHA/EPA may regulate fish immunity and disease resistance by altering the mRNA expression levels of TLR22 and MyD88.

1. Introduction

Many nutritional studies have demonstrated that marine fish need some n−3 highly unsaturated fatty acids (n−3 HUFA). These are mainly supplied by fish oils and meals, to maintain the normal body essential fatty acid composition and physiological functions (Glencross, 2009; Izquierdo et al., 2001; Kim and Lee, 2004; Kiron et al., 1995; Lavens et al., 1999; Lee and Cho, 2009; Montero et al., 2004; Skalli and Robin, 2004). Due to the low enzyme activities of Δ6 desaturase or elongase involved in the elongation–desaturation-chain short pathway, very limited amounts of eicosapentaenoic acid (EPA; 20:5n−3) can be converted into docosapentaenoic acid (DPA; 22:5n−3) *in vivo* (Sargent et al., 2002). This observation suggests that the relative proportion of EPA and DHA is equally important as the total n−3 HUFA when the different physiological role between these two kinds of fatty acids is considered (Glencross et al., 2011; Rodríguez et al., 1997; Wu et al., 2002, 2003). It is well known that neural tissue phospholipids of vertebrates are rich in DHA, which plays a critical role in visual and learning processes (Neuringer et al., 1984, 1988; Rodríguez et al., 1997). Thus, a diet lacking DHA or with a low ratio of DHA/EPA could result in the visual development problems which would then lead a decrease in hunting efficiency and consequently a reduction in growth rate of marine fish larvae (Mourente et al., 1993; Rodríguez et al., 1997; Watanabe et al., 1989a) and juveniles such as striped jack (Watanabe et al., 1989b), red seabream (Takeuchi et al., 1990), grouper (Wu et al., 2002) and barramundi (Glencross et al., 2011).

In addition, DHA/EPA could influence cellular functions of leukocytes in both humans and animals. *In vivo* studies showed that DHA-rich fish oil (FO) caused an increase in leukocyte functions such as phagocytosis, chemotactic response, and production rate of reactive oxygen species, while this phenomenon was not observed in leukocytes of volunteers

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with oral intake of EPA-rich FO (Gorjão et al., 2006, 2009; Kew et al., 2004; Miles et al., 2004). A relatively higher dietary DHA/EPA (2.0–3.0) could promote phagocytic functions, respiratory burst activities of grouper leucocytes and T-cell proliferation compared with low ratio (0.3–0.7) (Wu et al., 2003). On the other hand, solely supplementation of DHA has potentially adverse effects on host resistance to *Paracoccidioides brasiliensis* infection (Oarada et al., 2003) and T lymphocyte activation (Kew et al., 2004).

Mechanisms involved in modulation of the fish immunity by fatty acids are poorly understood and little is known about the regulation of fatty acids on the expression of immune related genes (Montero et al., 2008, 2010). Studies on human macrophages have showed that DHA and EPA had different impacts on the activation of nuclear factor kappa B (NF-κB) by altering the expression of the subunits as well as the inhibitory protein kappa B (IκB) and eventually the cytokine production (Gorjão et al., 2009; Weldon et al., 2007). However, as far as we know, no information was available about the effects of DHA/EPA on expression of some molecules linking the stimulus and the downstream signal transduction molecules, such as toll-like receptors (TLRs) and myeloid differentiation factor 88 (MyD88) in any species. In response to microbial intruders such as protozoa, bacteria, fungi, and viruses, TLRs mediate the activation of cell signaling cascades by MyD88-dependent or MyD88-independent pathway, ultimately resulting in the induction of the immune response and clearance of the microbial infection from host (Xiao et al., 2011; Yao et al., 2008, 2009).

Large yellow croaker, *Larmichthys crocea*, is an important marine fish species that is being widely cultured in southeast China. Studies on the nutrition of this fish have been conducted intensively in the past few years (Ai et al., 2004, 2007, 2008; Wang et al., 2010; Zhang et al., 2008), but little information is available on their lipid and fatty acid nutrition and subsequent effects on immune function. Due to the high-density culture of marine fish in floating sea cages and indoor rearing systems, white spot disease caused by the ciliate *Cryptocaryon irritans* may arise especially when water temperature stayed between 20 and 25 °C for a long time under which circumstances *C. irritans* could proliferate at a high speed (Martins et al., 2011; Sun et al., 2011; Watts et al., 2001). Thus, the present study was designed to determine the effects of DHA/EPA on growth, nonspecific immunological parameters, disease resistance to parasites as well as the expression of TLR22 and MyD88 in large yellow croaker following natural infestation of parasites.

2. Materials and methods

2.1. Feed ingredients and diet formulation

Five isoproteic (41% crude protein) and isolipidic (11% crude lipid) diets were formulated to contain graded ratios of DHA/EPA (0.61, 1.54, 2.17, 3.04 and 3.88) by adding different amounts of DHA-enriched oil (270.3 mg DHA and 6.5 mg EPA g^{-1} oil; both in the form of methylester; Hubei Youzhiyou Biotechnology Co., Ltd., Wuhan, China) and EPA-enriched oil (157.8 mg DHA and 301.2 mg EPA g^{-1} oil; both in the form of triglyceride; Hebei HAIYUAN Health Biological Science and Technology Co., Ltd., Cangzhou, China), and the ratio of 0.61 group was treated as the control group. Then some palmitin (palmitic acid content, 99.31% of total fatty acids; in the form of methylester; Shanghai Dinghua Chemical Co., Ltd., Shanghai, China) was added to a final amount of 7.0% oil mixture of dry weight. The total amount of n −3 HUFA was fixed at about 1.0% dry weight. White fish meal (crude protein 74.3% dry matter, crude lipid 6.6% dry matter) and soybean meal (crude protein 49.4% dry matter, crude lipid 0.9% dry matter) were chosen as the main protein sources. Ingredients and nutrient composition of the five experimental diets are given in details (Tables 1 and 2).

All ingredients were ground into fine powder such that they passed through a 320 μm screen. Ingredients of each diet were blended thoroughly first by hand and then machine. The oil mixture was then thoroughly mixed with all ingredients of each diet, after which, water (200 g kg^{-1}) was added to make stiff dough. Pellets (4 mm × 5 mm and 5 mm × 5 mm) were made using an automatic pellet-making machine (Weihai, Shandong province, China) and dried for about 12 h in a ventilated oven at 40 °C. After drying, feeds were packed in double plastic bags and stored at −15 °C until used.

Table 1
Formulation and proximate analysis of the experimental diets (% dry weight).

Ingredients	Dietary DHA/EPA ratio				
	0.61	1.54	2.17	3.04	3.88
White fish meal[a]	35.00	35.00	35.00	35.00	35.00
Soybean meal[a]	25.50	25.50	25.50	25.50	25.50
Wheat meal[a]	25.50	25.50	25.50	25.50	25.50
Mineral premix[b]	2.00	2.00	2.00	2.00	2.00
Vitamin premix[c]	2.00	2.00	2.00	2.00	2.00
Attractant[d]	0.30	0.30	0.30	0.30	0.30
Mold inhibitor[e]	0.10	0.10	0.10	0.10	0.10
Lecithin	2.60	2.60	2.60	2.60	2.60
DHA enriched oil[f]	0.10	1.15	1.78	2.20	2.52
EPA enriched oil[g]	1.68	1.05	0.67	0.42	0.25
Palmitin[h]	3.82	3.40	3.15	2.98	2.83
ARA enriched oil[i]	1.40	1.40	1.40	1.40	1.40
Proximate analysis (n = 3)					
Crude protein (%)	41.40	41.31	41.19	41.08	41.42
Crude lipid (%)	11.22	11.27	11.19	11.03	11.38
DHA/EPA ratio	0.61	1.54	2.17	3.04	3.88
n−3 HUFA (%)	1.07	1.04	1.02	1.04	1.03

[a] White fish meal: crude protein 74.3% dry matter, crude lipid 6.6% dry matter; soybean meal: crude protein 49.4% dry matter, crude lipid 0.9% dry matter; wheat meal: crude protein 16.4% dry matter, crude lipid 1.0% dry matter.

[b] Mineral premix (mg or g kg^{-1} diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; Ca $(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite, 18.35 g.

[c] Vitamin premix (mg or g kg^{-1} diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; folic acid, 20 mg; vitamin B_1, 25 mg; vitamin A, 32 mg; vitamin B_2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g.

[d] Attractant: glycine and betaine.

[e] Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

[f] DHA enriched oil: DHA content, 270.3 mg g^{-1} oil; EPA content, 6.5 mg g^{-1} oil; both in the form of DHA-methylester; Hubei Youzhiyou Biotechnology Co., Ltd., China.

[g] EPA enriched oil: EPA content, 301.2 mg g^{-1} oil; DHA content, 157.8 mg g^{-1} oil; both in the form of triglyceride; HEBEI HAIYUAN Health Biological Science and Technology Co., Ltd., China.

[h] Palmitin: Palmitic acid content, 99.3% of total fatty acids, in the form of methylester; Shanghai Dinghua Chemical Co., Ltd., China.

[i] ARA enriched oil: ARA content, 348.1 mg g^{-1} oil; in the form of ARA-methylester; Hubei Youzhiyou Biotechnology Co., Ltd., China.

2.2. Experimental procedure

Large yellow croaker were bought from a commercial farm in Xiangshan bay, Ningbo, China. Prior to the start of the experiment, juveniles were reared in floating sea cages (3 m × 3 m × 3 m) and fed the control diet for two weeks to acclimate to the experimental conditions and feeds.

At the start of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10,000) (Shanghai Reagent, China). Fish of similar sizes (9.8 ± 0.6 g; mean ± S.E.M.) were distributed into 15 sea cages (1 m × 1 m × 1.5 m), and each cage was stocked with 60 fish. Each diet was randomly allocated to triplicate cages of fish. Fish were hand-fed twice daily (05:00 and 17:00) to apparent satiation. The feeding trial lasted for 58 days. During the experimental period, the water temperature, salinity and dissolved oxygen were measured daily during the experimental period. The water temperature ranged from 21.5 to 30.0 °C, and salinity from 32‰ to 36‰. The dissolved oxygen was approximately 7 mg L^{-1}. At the termination of the experiment, the fish were fasted for 24 h before harvest. Total number and body weight of fish in each cage were measured.

Table 2
Fatty acid composition of the experimental diets (% total fatty acids).[a].

Fatty acid	Dietary DHA/EPA ratio				
	0.61	1.54	2.17	3.04	3.88
14:0	1.55	1.89	2.66	2.85	3.29
16:0	51.98	42.98	44.30	43.01	42.83
18:0	3.43	3.60	3.55	3.68	3.57
20:0	1.07	1.21	1.00	1.04	0.99
∑ SFA[b]	58.03	49.68	51.51	50.58	50.68
16:1	2.02	1.70	1.78	1.85	1.94
18:1	9.71	9.54	9.27	9.34	8.86
∑ MUFA[c]	11.73	11.24	11.05	11.19	10.80
18:2n−6	13.30	13.33	13.45	13.45	13.56
20:4n−6	4.58	5.51	5.18	5.25	5.29
∑ n−6 PUFA[d]	17.88	18.84	18.63	18.70	18.85
18:3n−3	1.41	1.45	1.48	1.45	1.57
20:5n−3	7.51	5.06	3.60	2.90	2.39
22:6n-3 4.58 7.79 7.82 8.81 9.27					
∑ n−3 PUFA[e]	13.50	14.30	12.90	13.16	13.23
n−3/n−6PUFA	0.76	0.76	0.69	0.70	0.70
n−3HUFA[f]	12.09	12.85	11.42	11.71	11.66
ARA/EPA[g]	0.61	1.09	1.44	1.81	2.21
DHA/EPA[h]	0.61	1.54	2.17	3.04	3.88

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n−9, C22:1n−11, C20:2n−6, C20:3n−6, C22:5n−3, were not listed in the table.
[b] SFA: saturated fatty acids.
[c] MUFA: mono-unsaturated fatty acids.
[d] n−6 PUFA: n−6 poly-unsaturated fatty acids.
[e] n−3 PUFA: n−3 poly-unsaturated fatty acids.
[f] n−3 HUFA: n−3 highly-unsaturated fatty acids.
[g] ARA/EPA: 20:4n−6/20:5n−3.
[h] DHA/EPA: 22:6n−3/20:5n−3.

2.3. Biochemical analysis

Crude protein was determined by digestion using the Kjeldahl method and estimated by multiplying nitrogen by 6.25. Crude lipid was measured by ether extraction using Soxhlet method. The fatty acid profiles were analyzed using the procedures described by Metcalfe et al. (1966) with some modification (Ai et al., 2008; Zuo et al., 2012). About 100 mg freeze-dried samples were added into a 20 ml volumetric screwed tube with cover. Then 3 ml potassium hydroxide methanol (1 N) was added and heated on 72°C water bath for 20 min. After that, 3 ml HCL–methanol (2 N) was added and the mixture was heated on 72°C water bath for another 20 min. Previous tests were conducted to make sure that all fatty acids can be esterified following the procedures above. Finally, 1 ml hexane was added into the mixture above, shaken vigorously for 1 min, and then allowed to separate into two layers. Fatty acid methyl esters were separated, and quantified by HP6890 gas chromatograph (Agilents Technologies Inc., Santa Clara, California, USA) with a fused silica capillary column (007-CW, Hewlett Packard, Palo Alto, CA, USA) and a flame ionization detector. The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C min^{-1}, from 200 °C to 250 °C at a rate of 2 °C min^{-1}. Injector and detector temperature was 250 °C, respectively.

2.4. Functional immune assay

2.4.1. Blood collection and serum separation

At the end of the feeding trial, large yellow croaker were fasted for 24 h and anesthetized with eugenol (1:10,000) (Shanghai Reagent, China). Blood samples were obtained from the caudal vein of five fish from each cage with 27-gauge needles and 1 ml syringes and allowed to clot at room temperature for 4 h and then at 4 °C for further 6 h. The clot was removed and residual blood cells separated from the straw-colored serum by centrifugation (836 *g*, 10 min, 4 °C). The serum was frozen in liquid nitrogen and then stored at −80 °C for later analysis of lysozyme and superoxide dismutase (SOD) activity.

2.4.2. Lysozyme activity

The lysozyme activity in serum was measured according to the method of Ellis (1990). Briefly, a sample of 0.05 ml serum was added to 1.4 ml of a suspension of *Micrococcus lysodeikticus* (Sigma) (0.2 mg ml^{-1}) in a 0.1 M sodium phosphate buffer (pH 6.8). The reaction was carried at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. Each unit is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute.

2.4.3. Superoxide dismutase (SOD) activity

Serum SOD activity was measured spectrophotochemically by the ferricytochrome *C* method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU ml^{-1} xanthine oxidase. The reaction was triggered by the addition of the xanthine oxidase. Results are expressed in units of SOD per milliliter serum and each unit is defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome *C* reduction rate measured at 550 nm (McCord and Fridovich, 1969).

2.4.4. Phagocytic index (PI)

PI was measured according to a method of Pulsford et al. (1995) with some modification. Head kidneys of 6 fish per cage were removed, homogenized in the modified formula of L-15 culture medium (10 μg ml^{-1} heparin; 200 U ml^{-1} penicillin/streptomycin; hepas 15 mmol L^{-1}; 1% foetal bovine serum) and then filtered through a 100 μm nylon mesh. The resulting cell suspensions were enriched by centrifugation (836 *g*, 25 min, 4 °C) on 34%/51% Percoll (Pharmacia, USA) density gradient. The cells were collected at the 34–51% interface and washed twice. The final cell concentration was adjusted to approximately 1×10^7 leucocytes ml^{-1} and the cell viability was more than 95%. Then 100 μl cell suspensions of head kidney leucocytes and 100 μl yeast suspension (Baker's yeast, Type II, Sigma, USA, 1×10^8 cells ml^{-1}) were mixed into a 2 ml sized plastic tube and cultured at 23.5 °C for 40 min. To calculate the PI, some mixture was put into the blood cell counting plate (Shanghai Qiujing Biochemical Reagent and Apparatus Co., Ltd., Shanghai, China) and 200 cells were counted where the number of cells with ingested yeasts was recorded.

2.4.5. Nitro blue tetrazolium (NBT) positive test

The NBT positive test was performed following a modified method of Walters and Narasimha Reddy (1974). NBT positive test in one cage were assayed in triplicate. A volume of 100 μl head kidney leucocytes (10^7 cells ml^{-1}) of 6 fish in one cage were mixed together with 100 μl NBT suspended in 0.9% PBS solution (1 mg ml^{-1}) and then the mixture was incubated in a 2 ml sized sterilized eppendorf tube for 15 min at 23.5 °C. Using blood cell counting plate, 200 cells were evaluated. NBT positive leucocytes showed a single dense of black–blue formazan deposit or a stippled cytoplasmic distribution of formazan, larger than the cell granules.

2.4.6. Natural infestation of parasites

At the day 58 of feeding experiment, experimental fish in this study showed significantly decreased appetite with visible white spots scattered on the body. Same events were reflected by many local farmers in this area almost at the same time. Experimental fish were confirmed to be infected with *C. irritans* according to morphological and molecular identification following the methods of Sun et al. (2006). Thus, the feeding experiment had to be ended which lasted for 58 days. Experimental fish were weighed and live fish number in each cage was counted to determine the survival rate during the 58 day feeding experiment. PI and NBT positive cell percentage were determined immediately and serum samples were taken, flash frozen in liquid nitrogen and then stored at −80 °C for the later analysis of

serum lysozyme and SOD activities. After that, 40 experimental fish were left in each cage to determine the cumulative mortality during the natural infestation of parasites in the following days. Three days later (early stage after natural parasite infestation), liver and kidney from five fish were pooled into 1.5 ml tube (RNAase-Free, Axygen, USA), frozen in liquid nitrogen and then stored at −80 °C for the analysis of immune related genes at the early stage after disease occurrence. Thirteen days later (later stage after natural parasite infestation), the observed highest cumulative mortality was about 70%. Thus, remaining live fish number in each cage was counted to calculate the actual cumulative mortality in 13 days following natural parasite infestation. At the same time, liver and kidney from five fish were collected, frozen in liquid nitrogen and then stored at −80 °C for the analysis of immune related gene expression at the late stage after disease occurrence.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from liver and kidney using Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with RNA-Free DNase (Takara, Japan) to remove DNA contaminant and reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) following the instructions. First strand cDNA was diluted by 4 times using sterilized double-distilled water. Real-time RT-PCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The amplification was performed in a total volume of 25 μl, containing 1 μl of each primer (10 μM), 1 μl of the diluted first strand cDNA product, 12.5 μl of 2× SYBR® Premix Ex Taq™II (Takara, Japan) and 9.5 μl of sterilized double-distilled water. The real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s. The primer sequence for β-actin, MyD88 and TLR22 were designed following the published sequences from large yellow croaker (Xiao et al., 2011; Yao et al., 2009) and listed in Table 5. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one 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 analyzed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiency was 1.020 for MyD88, 1.004 for β-actin and 0.9984 for LycTLR22. The abosolute ΔC_{T1} value (MyD88–β-actin) and ΔC_{T2} value (LycTLR22–β-actin) of the slope is 0.020 and 0.055, which are close to zero and indicate that $\Delta\Delta C_T$ calculation for the relative quantification of target genes can be used. To calculate the expression of MyD88 and LycTLR22, the comparative CT method ($2^{-\Delta\Delta t}$ method) was used as described by Yao et al. (2009).

2.6. Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate(SGR)} = (\text{Ln}W_t - \text{Ln}W_o) \times 100/t$$

$$\text{PI}(\%) = \text{Number of cells ingesting yeast} \times 100/\text{Number of head kidney leucocytes observed}$$

$$\text{NBT positive cell percentage}(\%) = \text{Number of NBT positive cells} \times 100/\text{Number of head kidney leucocytes observed}$$

$$\text{Survival rate}(\%) = N_{ft} \times 100/N_{fi}$$

$$\text{Cumulative mortality rate} = (N_o - N - N_t) \times 100/N_o$$

Where W_t and W_o were final and initial weight of fish during the 58 d feeding experiment, respectively; N_{fi} and N_{ft} were the initial and final fish number during the 58 d feeding experiment; N_o was the number of fish which were left in each cage after samples were taken for the assay of fatty acid and immune parameters; N was the number of fish in each cage which were sampled at the early and later stage following natural infestation of parasites; N_t was the number of remaining live fish in each cage after all samples were taken.

The statistical analysis was performed by using SPSS 16.0 for Windows. All data were subjected to a one-way analysis of variance (ANOVA) and differences between the means were tested by Tukey's multiple range test. The level of significance was chosen at $P<0.05$ and the results are presented as means ± S.E.M. (standard error of the mean).

Table 3
Growth response and survival of large yellow croaker fed diets with graded ratios of DHA/EPA.[a].

Immunological parameters	Dietary DHA/EPA ratio					Pooled S.E.M.[b]	*F*-value	*P*-value
	0.61	1.54	2.17	3.04	3.88			
Initial body weight (g)	9.79	9.79	9.79	9.79	9.79			
Final body weight (g)	31.79[a]	33.80[ab]	36.33[b]	35.95[ab]	35.51[ab]	0.59	3.56	0.04
SGR[c] (% d^{-1})	2.03[a]	2.14[ab]	2.26[b]	2.24[ab]	2.22[ab]	0.29	4.84	0.03
Survival (%)	96.4	97.9	93.0	93.8	96.4	0.80	1.33	0.34

[a] Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P<0.05$).
[b] S.E.M.: standard error of means.
[c] SGR: specific growth rate.

3. Results

3.1. Survival and growth

The survival rate was above 93.0% and there were no significant differences among dietary treatments in feeding trail ($P>0.05$). As dietary DHA/EPA increased from 0.61 to 2.17, the specific growth rate (SGR) increased significantly from 2.03% day^{-1} to 2.26% day^{-1} ($P<0.01$), and when DHA/EPA was equal to or above 3.04, the SGR was slightly lower than that of 2.17 group but still higher than the control group, though no significant differences were detected ($P>0.05$) (Table 3).

3.2. Fatty acid composition

Fatty acid composition of carcass, muscle and liver were listed in Tables 6, 7 and 8. EPA contents of carcass, muscle and liver decreased significantly with the increase of dietary DHA/EPA ($P<0.05$). While DHA increased significantly as dietary DHA/EPA increased ($P<0.05$). DHA/EPA and ARA/EPA in carcass, muscle and liver increased significantly with the increase of dietary DHA/EPA ratio ($P<0.01$). The n−3 HUFA concentration in muscle decreased significantly as dietary DHA/EPA increased ($P<0.05$). The n−3 HUFA concentration of muscle was lowest in fish fed diets with the highest DHA/EPA, significantly lower than that in other groups ($P<0.01$). However, no significant differences were found in the n−3 HUFA concentration of carcass and liver ($P>0.05$).

3.3. Immunological parameters

The serum lysozyme activity increased significantly from 145.73 to 188.93 units ml^{-1} with dietary DHA/EPA increased from 0.61 to 3.04 ($P<0.01$), and then significantly declined to 128.00 units ml^{-1} with further increase of this ratio ($P<0.01$). When dietary DHA/EPA increased from 0.61 to 2.17, NBT positive cell percentage increased

Table 4
Immune parameters of juvenile large yellow croaker fed diets with graded ratios of DHA/EPA.[a].

Immunological parameters	Dietary DHA/EPA ratio					Pooled S.E.M.[b]	*F*-value	*P*-value
	0.61	1.54	2.17	3.04	3.88			
Lysozyme activity (units ml^{-1})	145.73[a]	163.20[ab]	157.60[ab]	188.93[b]	128.00[a]	6.08	7.61	0.00
NBT positive cell percent[c](%)	52.10[a]	70.30[b]	83.98[c]	80.27[bc]	69.68[b]	3.46	29.66	0.00
SOD[d] activity (units ml^{-1})	79.43	82.02	78.42	76.32	81.33	1.21	0.64	0.64
PI[e] (%)	7.31	7.85	6.70	5.05	4.35	0.78	1.11	0.40

[a] Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P<0.05$).
[b] S.E.M.: standard error of means.
[c] NBT positive cell percent: nitro-blue tetrazolium chloride (NBT) positive macrophage percentage of head kidney.
[d] SOD: superoxide dismutase.
[e] PI: phagocytosis index.

significantly from 52.10% to 83.98% ($P<0.01$), before significantly decreased to 69.68% as DHA/EPA ratio increased from 2.17 to 3.88 ($P<0.01$). No significant differences were detected in PI and serum SOD activity among dietary treatments ($P>0.05$) (Table 4).

3.4. Cumulative mortality rate

The cumulative mortality rate following natural infestation of parasites significantly decreased from 80.8% to 58.8% when dietary DHA/EPA increased from 0.61 to 3.04 ($P<0.05$), and thereafter increased to 71.5% as this ratio increased from 3.04 to 3.88 ($P>0.05$). A second-order polynomial regression analysis based on cumulative mortality rate was used to estimate the optimal DHA/EPA ratio for juvenile large yellow croaker. The second-order polynomial regression curves [$Y=5.8216X^2-29.429X+97.036$ ($R^2=0.9627$)] was used to determine the optimal point for the lowest cumulative mortality rate (Fig. 1). The results showed that the optimal DHA/EPA ratio for the lowest mortality of juvenile large yellow croaker was 2.53.

3.5. Immune related gene expression

Relative mRNA expression of TLR22 and MyD88 at the early stage after natural infestation of parasites, *C. irritans* in the liver (A) and kidney (B) of large yellow croaker, *L. crocea* fed with graded dietary DHA/EPA were presented in Fig. 2. In liver, TLR22 expression levels significantly increased to the maximum levels when dietary DHA/EPA increased from 0.61 to 3.04 ($P<0.05$), and then decreased with DHA/EPA increased to 3.88 ($P>0.05$). The mRNA expression levels of TLR22 were increased by about 0.35-fold, 0.95-fold and 0.51-fold in the ratio of 2.17, 3.04 and 3.88 treatments. The expression levels of MyD88 transcript were up-regulated by 0.39-fold, 0.41-fold, 0.80-fold and 1.06-fold in the ratio of 1.54, 2.17, 3.04 and 3.88 treatments, respectively. MyD88 transcript levels in liver of fish fed diets with high DHA/EPA (3.04 and 3.88) were significantly higher than those of fish fed diets with DHA/EPA equal to or lower than 1.54 ($P<0.05$) (Fig. 2A). In kidney, TLR22 transcript levels were significantly up-regulated to the maximum (1.47-fold higher than the control group) ($P<0.05$), and then decreased as dietary DHA/EPA increased to 3.88. There was no significant difference in the expression levels of MyD88 among dietary treatments ($P>0.05$) (Fig. 2B).

Relative mRNA expression of TLR22 and MyD88 at the later stage after natural infestation of parasites, *C. irritans* in the liver (C) and kidney (D) of large yellow croaker, *L. crocea* fed with graded dietary DHA/EPA were presented in Fig. 3. The hepatic TLR22 transcript levels were down-regulated by 0.13-fold, 0.30-fold, 0.54-fold and 0.54-fold in the ratio of 1.54, 2.17, 3.04 and 3.88 treatments. The hepatic TLR22 expression levels in fish fed diets with the ratio of 3.04 and 3.08 were significantly lower than those of fish in the control group ($P<0.05$). The hepatic MyD88 expression significantly increased to the maximum (0.63-fold higher than the control group) as dietary DHA/EPA increased from 0.61 to 2.17 ($P<0.05$), and then decreased with increasing dietary DHA/EPA from 2.17 to 3.88 ($P>0.05$) (Fig. 3C). No significant differences were observed in the expression of TLR22 and MyD88 in kidney at the later stage after natural infestation of parasites ($P>0.05$) (Fig. 3D).

Table 5
qPCR primers used in this study.

Target gene	Reference	Forward (5′-3′)	Reverse(5′-3′)
TLR22[a]	Xiao et al. (2011)	AGCACCGACTTCATCTGCTTTG	TGGTCTTCCTGCTCGCATAGATG
MyD88[b]	Yao et al. (2009)	TCTCATTGCGCTCAACGTGA	CCATCGTCTCTGCAACAACCA
β-actin	Yao et al. (2009)	TTATGAAGGCTATGCCCTGCC	TGAAGGAGTAGCCACGCTCTGT

[a] TLR22: Toll-like receptor 22.
[b] MyD88: Myeloid differentiation factor 88.

Table 6
Fatty acid composition (% total fatty acids) in the carcass of juvenile large yellow croaker fed the diets with graded DHA/EPA.[a].

Fatty acid	Dietary DHA/EPA ratio					Pooled S.E.M.[b]	*F*-value	*P*-value
	0.61	1.54	2.17	3.04	3.88			
14:0	2.71[a]	3.16[b]	2.94[ab]	3.10[ab]	4.09[c]	0.15	44.16	0.00
16:0	33.39	37.95	33.04	35.10	36.59	0.66	3.32	0.06
18:0	5.72	6.06	5.61	5.78	5.47	1.00	1.08	0.42
20:0	2.34[a]	1.95[ab]	2.38[a]	1.68[b]	1.69[b]	0.09	9.87	0.00
∑SFA[c]	44.15	49.28	44.30	45.68	47.84	0.73	2.90	0.08
16:1	5.44	5.61	5.25	4.82	5.42	0.11	1.81	0.21
18:1	16.64	16.70	16.62	15.94	15.32	0.20	2.96	0.08
∑MUFA[d]	22.08	22.31	21.87	20.76	20.74	0.26	2.19	0.14
18:2n−6	9.74[ab]	10.38[ab]	9.17[a]	11.27[b]	10.88[b]	0.24	6.28	0.01
20:4n−6	3.92	4.08	3.90	4.37	4.22	0.07	1.68	0.23
∑n−6 PUFA[e]	13.66[ab]	14.46[ab]	13.07[a]	15.64[b]	15.10[b]	0.30	5.79	0.01
18:3n−3	1.13	1.07	1.11	1.21	1.23	0.03	1.13	0.42
20:5n−3	4.53[a]	3.28[b]	3.17[b]	2.59[c]	2.33[c]	0.21	50.58	0.00
22:6n−3	7.24	6.44	8.50	8.31	8.13	0.27	3.51	0.05
∑n−3 PUFA[f]	12.89	10.43	13.09	12.11	11.28	0.35	3.47	0.05
n−3/n−6PUFA	0.84	0.72	0.80	0.77	0.75	0.03	1.45	0.41
n−3HUFA[g]	11.76	9.72	11.67	10.89	10.46	0.29	2.45	0.11
ARA/EPA[h]	0.87[a]	1.24[b]	1.24[b]	1.69[c]	1.82[c]	1.00	30.18	0.00
DHA/EPA[i]	1.59[a]	1.96[a]	2.69[b]	3.21[bc]	3.50[c]	0.20	36.25	0.00

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n−9, C22:1n−11, C20:2n−6, C20:3n−6, C22:5n−3, were not listed in the table. Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P<0.05$).
[b] S.E.M.: standard error of means.
[c] SFA: saturated fatty acids.
[d] MUFA: mono-unsaturated fatty acids.
[e] n−6 PUFA: n−6 poly-unsaturated fatty acids.
[f] n−3 PUFA: n−3 poly-unsaturated fatty acids.
[g] n−3 HUFA: n−3 highly-unsaturated fatty acids.
[h] ARA/EPA: 20:4n−6/20:5n−3.
[i] DHA/EPA: 22:6n−3/20:5n−3.

Table 7
Fatty acid composition (% total fatty acids) in the muscle of juvenile large yellow croaker fed the diets with graded DHA/EPA.[a].

Fatty acid	Dietary DHA/EPA ratio					Pooled S.E.M.[b]	*F*-value	*P*-value
	0.61	1.54	2.17	3.04	3.88			
14:0	2.84[a]	2.79[a]	2.72[a]	2.92[ab]	3.43[b]	0.08	5.18	0.02
16:0	29.60[a]	30.89[ab]	30.14[ab]	30.77[ab]	33.41[b]	0.46	3.62	0.04
18:0	5.22	5.43	5.49	5.03	5.15	0.09	0.98	0.46
20:0	2.09[a]	2.00[ab]	1.90[ab]	1.72[bc]	1.50[c]	0.06	10.94	0.00
∑SFA[c]	39.74	41.11	40.25	40.44	43.50	0.49	2.77	0.09
16:1	5.14	4.90	4.55	4.48	5.09	0.10	2.67	0.09
18:1	14.20[a]	16.59[b]	16.16[b]	15.17[ab]	15.72[ab]	0.27	5.61	0.01
∑MUFA[d]	19.34	21.49	20.71	19.65	20.81	0.29	2.71	0.09
18:2n−6	9.16[a]	9.44[ab]	10.02[abc]	11.14[c]	10.61[bc]	0.22	8.16	0.00
20:4n−6	4.13[a]	4.07[a]	4.43[ab]	4.85[b]	4.27[ab]	0.09	5.44	0.01
∑n−6 PUFA[e]	13.29[a]	13.51[a]	14.45[ab]	15.99[b]	14.88[ab]	0.30	7.21	0.01
18:3n−3	1.19[a]	1.20[a]	1.19[a]	1.31[b]	1.24[ab]	0.01	6.27	0.01
20:5n−3	5.41[a]	4.26[b]	3.50[c]	3.27[c]	2.58[d]	0.26	258.15	0.00
22:6n−3	8.58[a]	8.92[ab]	9.82[cd]	10.66[d]	9.64[bc]	0.21	20.36	0.00
∑n−3 PUFA[f]	15.18[a]	14.38[a]	14.52[a]	15.24[a]	13.46[b]	0.19	12.96	0.00
n−3/n−6PUFA	1.15[a]	1.07[ab]	1.01[ab]	0.95[ab]	0.90[b]	0.03	5.24	0.02
n−3HUFA[g]	13.99[a]	13.18[a]	13.33[a]	13.93[a]	12.22[b]	0.19	13.05	0.00
ARA/EPA[h]	0.76[a]	0.96[a]	1.27[b]	1.48[bc]	1.66[c]	0.09	49.60	0.00
DHA/EPA[i]	1.59[a]	2.09[b]	2.81[c]	3.26[d]	3.75[e]	0.21	102.86	0.00

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n−9, C22:1n−11, C20:2n−6, C20:3n−6, C22:5n−3, were not listed in the table. Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P<0.05$).
[b] S.E.M.: standard error of means.
[c] SFA: saturated fatty acids.
[d] MUFA: mono-unsaturated fatty acids.
[e] n−6 PUFA: n−6 poly-unsaturated fatty acids.
[f] n−3 PUFA: n−3 poly-unsaturated fatty acids.
[g] n−3 HUFA: n−3 highly-unsaturated fatty acids.
[h] ARA/EPA: 20:4n−6/20:5n−3.
[i] DHA/EPA: 22:6n−3/20:5n−3.

4. Discussion

Studies on marine fish larvae have shown that more DHA is needed than EPA as the essential fatty acid (Izquierdo et al., 1989; Mourente et al., 1993; Watanabe et al., 1989a). However, there is little information available on the appropriate DHA/EPA for growth of juvenile marine fish. In the present study, the SGR of fish fed diets with a relatively higher DHA/EPA ratio was greater than that in the control and low ratio treatments, though significant differences were detected only between the ratio of 2.17 and the control group. This observation indicated that DHA was more optimal than EPA in promoting the growth of juvenile large yellow croaker as observed in marine fish larvae. It has been established that DHA is preferentially retained in neural tissues such as brain and eyes and crucial for effective prey capture from the time of first feeding (Bell et al., 1995; Morais et al., 2011; Mourente and Tocher, 2009). Furthermore, dietary arachidonic acid (ARA) to EPA ratio increased with enhanced ratio of dietary DHA/EPA as in this study, and more prostaglandin E_2 (PGE_2) from ARA was probably produced due to the less competitive effect of EPA on cyclooxygenase and lipoxygenase as ARA/EPA increased (Gorjão et al., 2009; Lo et al., 1999). PGE_2 was found to have stimulatory effects on bone growth in mammals (Appel et al., 1993; Ueno et al., 1985). Thus, different physiological role and metabolism pathway between DHA and EPA could be responsible for the requirement of a higher dietary DHA/EPA for large yellow croaker.

It is widely known that EPA exerts a stronger inhibitory effect on the lymphocyte proliferation in mammals than DHA (Fowler et al., 1993) and DHA in the absence of EPA does not suppress lymphocyte function (Kelley and Rudolph, 2000). In accordance, the present study showed that fish in the control group had a relatively lower nonspecific immunity than those fed the diet with a moderate or higher ratio of DHA/EPA, which was reflected by the selected parameters including serum lysozyme activity and NBT positive cell percent. The NBT-positive cell percentage is the proportion of leucocytes with dark blue formazan and

Table 8
Fatty acid composition (% total fatty acids) in the liver of juvenile large yellow croaker fed the diets with graded DHA/EPA.[a].

Fatty acid	Dietary DHA/EPA ratio					Pooled S.E.M.[b]	*F*-value	*P*-value
	0.61	1.54	2.17	3.04	3.88			
14:0	1.49[a]	2.15[ab]	1.96[ab]	2.18[ab]	2.58[b]	0.12	4.21	0.03
16:0	33.51[ab]	36.30[a]	34.08[ab]	30.87[b]	31.48[b]	0.62	5.97	0.01
18:0	5.62	7.99	6.68	5.38	5.84	0.36	2.58	0.10
20:0	1.33[ab]	1.41[a]	1.17[b]	1.21[b]	1.27[b]	0.03	3.96	0.04
∑SFA[c]	41.93[a]	47.38[b]	43.88[ab]	39.64[a]	41.17[a]	0.81	8.28	0.00
16:1	4.77	5.08	4.33	4.57	6.20	0.29	1.35	0.32
18:1	13.26	13.59	13.51	13.67	13.57	0.29	0.05	1.00
∑MUFA[d]	18.02	19.38	17.84	18.25	19.77	0.48	0.56	0.70
18:2n−6	16.53[a]	13.42[b]	15.24[ab]	15.56[ab]	14.47[ab]	0.35	5.13	0.02
20:4n−6	5.53	5.56	5.29	5.55	6.06	0.22	0.73	0.60
∑n−6 PUFA[e]	22.06	18.98	18.50	21.11	20.53	0.61	1.31	0.34
18:3n−3	1.23	1.17	1.32	1.27	1.29	0.07	12.15	0.00
20:5n−3	2.83[a]	1.93[b]	1.94[b]	1.90[b]	1.19[b]	0.16	13.60	0.00
22:6n−3	5.51[a]	6.72[ab]	6.07[ab]	8.64[b]	9.19[b]	0.50	4.81	0.02
∑n−3 PUFA[f]	9.57	9.22	10.18	11.81	11.27	0.40	2.18	0.16
n−3/n−6PUFA	0.44	0.48	0.50	0.56	0.55	0.02	3.71	0.04
n−3HUFA[g]	8.34	8.44	8.01	10.54	10.38	0.44	2.20	0.15
ARA/EPA[h]	1.95[a]	2.22[ab]	2.54[b]	2.96[b]	5.76[c]	0.35	72.83	0.00
DHA/EPA[i]	1.96[a]	3.16[b]	3.90[c]	4.59[d]	8.81[e]	0.64	81.01	0.00

[a] Some fatty acids, of which the contents are minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n−9, C22:1n−11, C20:2n−6, C20:3n−6, C22:5n−3, were not listed in the table. Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P<0.05$).
[b] S.E.M.: standard error of means.
[c] SFA: saturated fatty acids.
[d] MUFA: mono-unsaturated fatty acids.
[e] n−6 PUFA: n−6 poly-unsaturated fatty acids.
[f] n−3 PUFA: n−3 poly-unsaturated fatty acids.
[g] n−3 HUFA: n−3 highly-unsaturated fatty acids.
[h] ARA/EPA: 20:4n−6/20:5n−3.
[i] DHA/EPA: 22:6n−3/20:5n−3.

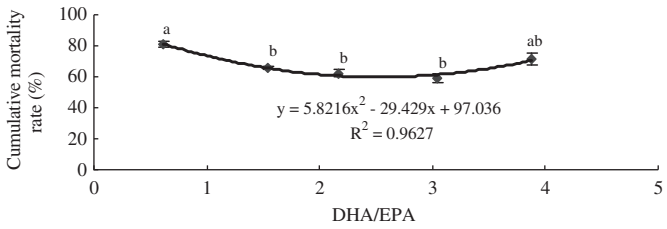

Fig. 1. The 13 day cumulative mortality of juvenile large yellow croaker among different dietary groups following natural infestation of parasites, *Cryptocaryon irritans*. Values are means ± S.E.M. (n = 3). Bars bearing with different letters are significantly different by Tukey's test ($P<0.05$).

thus can be taken as a capacity of leucocytes to produce intracellular reactive oxygen species (ROS). NBT-positive cell percentage and SOD play a critical role in controlling the balance of release and clear of ROS in immune cells, particularly during the immune processes such as phagocytosis, and hence are indicative parameters of immune functions (Campa-Córdova et al., 2002; Holmblad and Söderhäll, 1999; Xu et al., 2010). A previous study found that phagocytic function was enhanced by a higher DHA/EPA ratio (Wu et al., 2003). However, PI showed no significant differences among dietary treatments in the present study, which could be due to immune fatigue caused by the parasite infection. This was confirmed by the findings of Sitjà-Bobadilla et al. (2008) who also found that total serum peroxidases and lysozyme activity of gilthead sea bream could be initially increased in response to parasite exposure, but was quickly exhausted in response to it, and did not recover even in the uninfected fish.

In the present study, expression of TLR22 and MyD88 was significantly influenced by dietary DHA/EPA ratio and sampling time. At the early parasite infection stage, TLR22 mRNA expression in liver and kidney showed a similar changing trend with the increase of dietary DHA/EPA ratio and the highest value was found in the ratio of 3.04 and 2.17, respectively. However, relative hepatic mRNA expression of MyD88 increased significantly as dietary DHA/EPA ratio increased

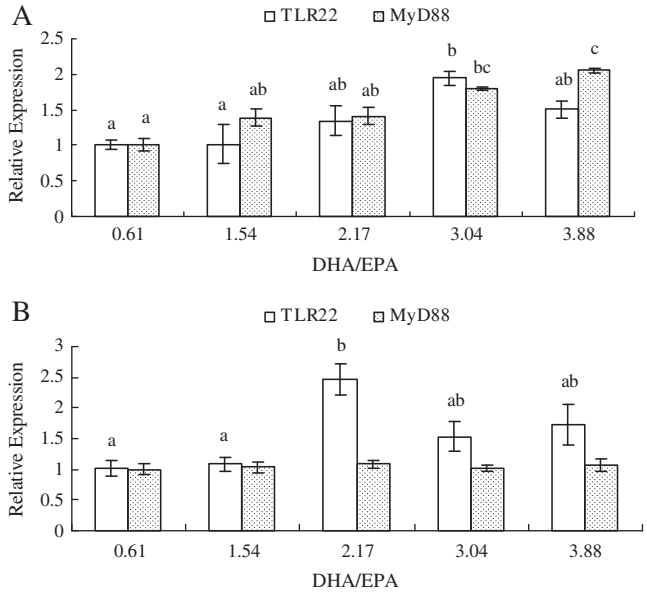

Fig. 2. Relative mRNA expression of TLR22 and MyD88 at the early stage after natural infestation of parasites, *Cryptocaryon irritans* in the liver (A) and kidney (B) of large yellow croaker, *Larmichthys crocea* fed with graded DHA/EPA for 58 days. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.E.M. (n = 3). Bars of the same gene bearing with different letters are significantly different by Tukey's test ($P<0.05$).

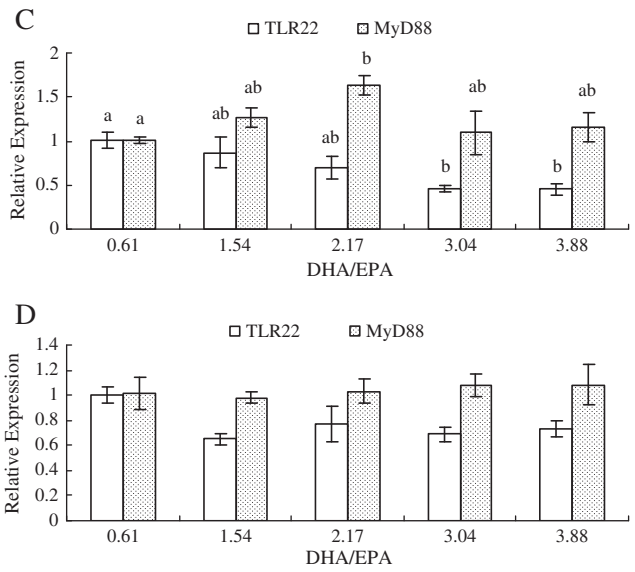

Fig. 3. Relative mRNA expression of TLR22 and MyD88 at the later stage after natural infestation of parasites, *Cryptocaryon irritans* in the liver (C) and kidney (D) of large yellow croaker, *Larmichthys crocea* fed with graded DHA/EPA for 58 days. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± S.E.M. (n = 3). Bars of the same gene bearing with different letters are significantly different by Tukey's test ($P<0.05$).

with no inhibitory effect found in the high ratio, which suggested that different genes could respond differently to the ratio of DHA/EPA. The expression of TLR22 and MyD88 seems to be positively correlated with the intracellular ROS production, reflected by the parameter of NBT positive cell percentage of head kidney. After bacteria are ingested by phagocytes, ROS exert a critical role in bacterial killing (Sharp and Secombes, 1993; Sharp et al., 1991) and evidences have shown the importance of TLR4 in the ROS production. *In vitro* studies on kidney epithelial cells have shown prolonged release of ROS due to TLR4 after stimulation with heat-killed uropathogenic *Escherichia coli* GR-12 (Zhao et al., 2010). Moreover, a genetic defect in TLR4 could be responsible for the impairment of neutrophils in generating superoxide anion in response to LPS (Remer et al., 2003) and ROS production after vascular injury was attenuated in TLR2 knockout mice compared with control littermates (Shishido et al., 2006). This may reflect the importance of TLRs during early disease recognition, whose lost or low expression may decrease the sensitivity of disease recognition by the host.

Apart from the benefit for the enhancement of the nonspecific immunity, TLR22 and MyD88 were also involved in the inflammation process which may be responsible for the tissue injury under infective or oxidative stress as observed by some workers (Aprahamian et al., 2008; Chen et al., 2009; Gill et al., 2010; Kilic et al., 2008; Shigeoka et al., 2007; Tsung et al., 2005; Wu et al., 2007; Zhai et al., 2004). In this study, hepatic relative expression of TLR22 and MyD88 was decreased in the high DHA/EPA treatment on 13 day post parasite infection, which indicated that high DHA/EPA may benefit large yellow croaker through decreasing TLR22 and MyD88 expression to deal with serious inflammation responses during the process of parasite infective stress. The anti-inflammatory effect of n−3 LC-PUFA has been observed in a lot of studies (Dröge, 2002; Grimble et al., 2002; Weatherill et al., 2005) and results in this study showed that DHA could be more effective than EPA in controlling the inflammation by decreasing TLR22 and MyD88 expression which was consistent with some previous studies (Pisani et al., 2009; Weldon et al., 2007) while inconsistent with the findings of Mullen et al. (2010) who found both DHA and EPA had the same anti-inflammatory effects.

This was probably be due to different experiment subjects, conditions and parameters selected and needs more investigation in the future.

In this study, fish had a significantly lower cumulative mortality fed the diets with a ratio of DHA/EPA between 1.54 and 3.04. And the optimal DHA/EPA ratio was 2.53 using the second-order polynomial regression analysis based on cumulative mortality rate. In accordance, nonspecific immunity and the expression of TLR22 and MyD88 at the early stage of parasite infection were higher in the ratio of 2.17 and 3.04 treatments, which seems to indicate that lower cumulative mortality may be due to enhanced innate immunity and alert to invading disease. Previous studies have shown that TLR2 and TLR4 mRNA expression was upregulated in cecum and spleen in response to *Salmonella enteritidis* infection in young chicks (Abasht et al., 2008) and MyD88-deficient mice are more susceptible to many different microbial pathogens (Feng et al., 2003; Kawai et al., 1999; Seki et al., 2002; Takeuchi et al., 2000), suggesting the important role of TLRs and MyD88 in the disease recognition and defenses.

In conclusion, the results of the present study show that a higher ratio of DHA/EPA (2.17–3.04) could significantly improve growth performance, nonspecific immunity, provide enhanced protection against parasite infection, as well as affect hepatic and kidney mRNA expression of TLR22 and MyD88 at the early parasite infection stage in juvenile large yellow croaker. Further information is required to determine the consequences of DHA/EPA ratio on other components of TLR signaling pathway, such as proinflammatory cytokines, at mRNA and protein levels to understand how dietary DHA/EPA ratio modulates the immune system of marine fish.

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