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Effects of dietary n-3 highly unsaturated fatty acids 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 n-3 highly unsaturated fatty acid (n-3 HUFA) on growth, nonspecific immunity, expression of some immune related genes and disease resistance of juvenile large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). Six isoproteic and isolipidic diets were formulated with graded levels of n-3 HUFA ranging from 0.15% to 2.25% of the dry weight and the DHA/EPA was approximately fixed at 2.0. 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 9.79 ± 0.6 g). Fish were fed twice daily (05:00 and 17:00) to apparent satiation for 58 days. Results showed that moderate n-3 HUFA level (0.98%) significantly enhanced growth compared with the control group (0.15% HUFA) ($P < 0.05$), while higher n-3 HUFA levels (1.37%, 1.79% and 2.25%) had detrimental effects on the growth though no significance was found ($P > 0.05$). Nitro blue tetrazolium (NBT) positive leucocytes percentage of head kidney and serum superoxide dismutase (SOD) activity increased with increasing n-3 HUFA from 0.15% to 0.60%, and decreased with further increase of n-3 HUFA from 0.60% to 2.25% ($P < 0.05$). Serum lysozyme activity increased significantly as n-3 HUFA increased from 0.15% to 1.37%, and then decreased with n-3 HUFA from 1.37% to 2.25% ($P > 0.05$). There were no significant differences in phagocytosis index (PI) of head kidney leucocytes among dietary treatments ($P > 0.05$). The hepatic mRNA expression of Toll-like receptor 22 (TLR22) and Myeloid differentiation factor 88 (MyD88) was significantly up-regulated in fish fed the diets with low or moderate levels, while in kidney this increment was only found at specific sampling time during the natural infestation of parasites. The 13 d cumulative mortality rate following natural infestation of parasites decreased with n-3 HUFA increased from 0.15% to 0.60% ($P < 0.05$), and significantly increased with n-3 HUFA from 0.60% to 2.25% ($P < 0.05$). Results of this study suggested that fish fed low or moderate dietary n-3 HUFA had higher growth, nonspecific immune responses, expression levels of some immune related genes and disease resistance of large yellow croaker following natural infestation of parasites and dietary n-3 HUFA may regulate fish immunity and disease resistance by altering the mRNA expression levels of TLR22 and MyD88.

1. Introduction

It is acknowledged that marine fish lack capacity to elongate and further desaturate linolenic acid (LNA; 18:3n-3) into n-3 highly unsaturated fatty acids (n-3 HUFA) *de novo*, mainly eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [1,2]. Therefore, some exogenous n-3 HUFA need to be provided in marine fish diets and in-adequate supply can affect fish growth [3–5], immunity [6,7], as well as unbalanced nutrition of broodstock and eventually the normal reproduction and survival of offspring [5,8,9]. On the other hand, high dietary n-3 HUFA also had detrimental effects on fish growth [4], immunity [10,11] and egg quality of broodstock [12]. Up to now, n-3 HUFA are largely supplied by fish oil and a few alternative oil sources derived from unicellular algae, pelagic organisms and benthic invertebrates among all the available lipid sources [13]. With increasing demand for fish oil and decreasing supplies of marine meals and oils, it is necessary to

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determine the requirement for n-3 HUFA in order to use fish-derived n-3 HUFA more efficiently.

Although many studies have covered the modulation of the fish immune system induced by n-3 HUFA [6,7,10,11], very little is known about the role of dietary fatty acids as modulator of the expression of certain genes involved in immune response and disease resistance. Recently, Montero et al. [14,15] has provided first evidences about the effect of vegetable oils in expression of genes related with protein of resistance against mixovirus (Mx protein), tumor necrosis factor-α (TNF-α) and interleukine 1β (IL-β) in marine fish. However, as far as we know, no information is available on the effects of dietary fatty acids on gene expression related with pathogen recognition and signal transduction such as toll-like receptors (TLRs) and myeloid differentiation factor 88 (MyD88) in any fish species, which play pivotal role in initiation of innate immune responses. TLRs are a family of transmembrane receptors which can recognize conserved pathogen-associated molecular patterns (PAMPs) [16,18]. MyD88 is an adaptor protein which can receive signals from TLRs and interleukin-1 receptor, and thereafter activate nuclear factor-kappaB (NF-κB) [17]. 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 [16–18].

Large yellow croaker, *Larmichthys crocea*, is an important marine fish species that has been widely cultured in southeast China. Studies on nutrition of this fish have been conducted intensively in the past few years [19–23], but no information is available on its lipid and fatty acid nutritional immunity. 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 burst out 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 [24–26]. Thus, the present study was designed to determine the effects of dietary n-3 HUFA on growth, nonspecific immunological parameters, expression of some immune related genes (TLR22 and MyD88) and disease resistance in large yellow croaker following natural infestation of parasites.

2. Materials and methods

2.1. Feed ingredients and diet formulation

Ingredients and nutrient composition of the experimental diets are given in Tables 1 and 2. Six isoproteic (41.3% crude protein) and isolipidic (11.7% crude lipid) diets were formulated to contain graded levels of n-3 HUFA (0.15, 0.60, 0.98, 1.37, 1.79 and 2.25% dry weight) by supplementation 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 0.15% HUFA group was treated as the control group. Then different amount of 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 total oil mixture amount of 7.5% of dry weight. Defatted fish meal, soybean meal and casein were used as main protein sources. Defatted fish meal (crude protein 79.1% dry matter, crude lipid 1.6% dry matter) was made by mixing the white fish meal (crude protein 74.3% dry matter, crude lipid 6.6% dry matter) with ethanol (1:2, w/v) and defatted at 37 °C in a 4 L plastic bucket for three times.

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

Ingredients (%)	Dietary n-3 HUFA contents (% dry weight)					
	0.15	0.60	0.98	1.37	1.79	2.25
Defatted white fish meal[a]	15.00	15.00	15.00	15.00	15.00	15.00
Soybean meal	32.00	32.00	32.00	32.00	32.00	32.00
Casein[b]	12.00	12.00	12.00	12.00	12.00	12.00
Wheat meal	25.50	25.50	25.50	25.50	25.50	25.50
Mineral premix[c]	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[d]	2.00	2.00	2.00	2.00	2.00	2.00
Attractant	0.30	0.30	0.30	0.30	0.30	0.30
Mold inhibitor	0.10	0.10	0.10	0.10	0.10	0.10
Lecithin	2.60	2.60	2.60	2.60	2.60	2.60
DHA-enriched oil[e]	0.05	0.77	1.48	2.18	2.93	3.62
EPA-enriched oil[f]	0.00	0.45	0.90	1.36	1.79	2.26
Palmitin[g]	7.45	6.28	5.12	3.96	2.78	1.62
ARA enriched oil[h]	1.00	1.00	1.00	1.00	1.00	1.00
Total	100	100	100	100	100	100
Proximate analysis ($n = 3$)						
Crude protein (%)	41.27	41.21	40.99	42.08	41.42	41.36
Crude lipid (%)	11.37	11.37	11.29	11.03	11.18	10.98
n-3 HUFA contents (% dry weight)	0.15	0.60	0.98	1.37	1.79	2.25

[a] Defatted fish meal: 79.1% crude protein and 1.6% crude lipid; white fish meal were defatted with ethanol (fish meal:ethanol = 1:2 (w:v)) at 37 °C for three times.
[b] Casein: 93% crude protein and 1% crude lipid, Alfa Aesar, Avocado Research Chemicals Ltd, UK.
[c] 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.
[d] 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.
[e] DHA-enriched oil: DHA content, 270.3 mg g^{-1} oil; EPA content, 6.5 mg g^{-1} oil; both in the form of methylester; Hubei Youzhiyou Biotechnology Co., Ltd., China.
[f] 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.
[g] Palmitin: Palmitic acid content, 99.3% of TFA, in the form of methylester; Shanghai Dinghua Chemical Co., Ltd., China.
[h] ARA enriched oil: ARA content, 348.1 mg g^{-1} oil; in the form of ARA-methylester; Hubei Youzhiyou Biotechnology Co., Ltd., China.

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

Fatty acid	Dietary n-3 HUFA contents (% dry weight)					
	0.15	0.60	0.98	1.37	1.79	2.25
14:0	0.57	1.03	1.79	2.23	2.80	3.53
16:0	70.55	64.25	60.40	48.67	39.77	31.16
18:0	2.71	2.67	2.58	2.75	2.87	2.91
20:0	0.43	0.48	0.49	0.66	0.73	0.84
∑SFA[a]	74.26	68.43	65.26	54.30	46.18	33.43
16:1	0.77	0.73	0.78	0.77	0.82	0.86
18:1	5.99	6.15	6.34	6.90	7.14	7.60
∑MUFA[b]	6.76	6.88	7.12	7.67	7.96	8.46
18:2n-6	12.27	12.74	12.87	13.81	13.99	14.53
20:4n-6	3.37	3.65	3.46	4.23	4.39	4.61
∑n-6 PUFA[c]	15.64	16.40	16.33	18.04	18.38	19.14
18:3n-3	1.27	1.31	1.34	1.47	1.50	1.64
20:5n-3	0.48	1.71	2.79	4.86	6.32	8.05
22:6n-3	0.91	3.54	5.00	9.44	12.68	15.71
∑n-3 PUFA[d]	2.66	6.56	9.14	15.78	20.51	25.39
n-3/n-6PUFA	0.17	0.40	0.56	0.87	1.11	1.33
n-3HUFA[e]	1.39	5.25	7.79	14.31	19.01	23.75
DHA/EPA[f]	1.90	2.02	1.93	1.94	2.01	1.95

[a] SFA: saturated fatty acids.
[b] MUFA: mono-unsaturated fatty acids.
[c] n-6 PUFA: n-6 poly-unsaturated fatty acids.
[d] n-3 PUFA: n-3 poly-unsaturated fatty acids.
[e] n-3 HUFA: n-3 highly unsaturated fatty acids.
[f] DHA/EPA: 22:6n-3/20:5n-3.

Table 3
Growth response and survival of large yellow croaker fed diets with graded levels of n-3 HUFA.[a]

	Dietary n-3 HUFA contents (% dry weight)						*F*-value	*P*-value
	0.15	0.60	0.98	1.37	1.79	2.25		
Initial body weight (g)	9.79 ± 0.6	9.79 ± 0.6	9.79 ± 0.6	9.79 ± 0.6	9.79 ± 0.6	9.79 ± 0.6		
Final body weight (g)	28.22 ± 0.76a	32.82 ± 0.24ab	36.04 ± 2.02b	31.18 ± 0.50ab	31.10 ± 0.68ab	31.11 ± 1.30ab	5.996	0.008
SGR (% d^{-1})[b]	1.82 ± 0.05a	2.09 ± 0.01ab	2.24 ± 0.10b	2.00 ± 0.03b	1.99 ± 0.04b	1.99 ± 0.07b	5.877	0.009
Feed intake (g)	1126.7 ± 54.4a	1434.4 ± 47.5bc	1542.8 ± 22.2c	1514.5 ± 50.5bc	1279.3 ± 46.8ab	1315.5 ± 22.5ab	11.91	0.002
Survival (%)	93.2 ± 3.1	95.8 ± 1.9	96.9 ± 1.56	94.8 ± 1.4	93.2 ± 3.2	91.1 ± 0.5	0.779	0.585

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

All ingredients were ground into fine powder through 320 μm mesh. Ingredients in one diet were blended thoroughly first by hand and then machine. After that, oil mixture was thoroughly mixed with all ingredients in one diet. At last, water was incorporate to make stiff dough. Pellets (4 mm × 5 mm and 5 mm × 5 mm) were made automatically by 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 in big nylon bags at room temperature until use.

2.2. Experimental procedure

Large yellow croaker was bought from a local fish rearing farm in Xiangshan bay, Ningbo, China. Prior to the start of the experiment, fish with similar size were reared in floating sea cages (3 m × 3 m × 3 m) and fed the control diet twice daily (05:00 and 17:00) for two weeks to adapt 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). Sixty fish of similar sizes (9.79 ± 0.6 g) were distributed into each sea cage (1 m × 1 m × 1.5 m), and each diet was randomly allocated to triplicate groups of fish. Fish were fed twice daily (05:00 and 17:00) to apparent satiation for 58 days. The water temperature, salinity and dissolved oxygen were detected everyday 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.

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. [27] with some modification [21]. About 100 mg freeze-dried samples were added into a 20 ml volumetric screwed glass tube with plastic 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 test has been conducted to make sure that all fatty acids can be esterified following the procedures above. At last, 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 in the upper layer 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 were 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 [28]. 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 a 50% inhibition of the ferricytochrome *C* reduction rate measured at 550 nm [29].

2.4.4. Phagocytic index (PI)

PI was measured according to a method of Pulsford et al. [30] 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 for 25 min at 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 (Bakers yeast, Type II, Sigma, USA, 1×10^8 cells ml^{-1}) were mixed into a 2 ml sized sterilized eppendorf 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 et al. [31]. 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 58 d 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. [32]. 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 d 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. Then, 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 [17,18] and listed in Table 4. 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. [17] (Table 5).

2.6. Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate(SGR)} = \frac{(\text{Ln}W_t - \text{Ln}W_0)}{t} \times 100$$

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

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

Table 4
Immune parameters of juvenile large yellow croaker fed diets with graded levels of n-3 HUFA.[a]

	Dietary n-3 HUFA contents (% dry weight)						*F*-value	*P*-value
	0.15	0.60	0.98	1.37	1.79	2.25		
Lysozyme activity (units ml^{-1})	143.20 ± 12.87a	157.47 ± 1.8a	182.00 ± 8.40ab	203.20 ± 7.6b	166.67 ± 12.8ab	173.60 ± 4.87ab	5.241	0.011
NBT positive cell percent[b] (%)	46.61 ± 3.49a	75.04 ± 3.21b	61.75 ± 4.33ab	62.52 ± 9.58ab	59.78 ± 2.85ab	55.46 ± 2.55ab	3.555	0.033
SOD activity (units ml^{-1})[c]	73.54 ± 2.39a	99.46 ± 1.22b	74.70 ± 4.86a	77.31 ± 2.50ab	75.41 ± 2.52a	83.95 ± 6.59ab	4.038	0.034
PI (%)[d]	4.97 ± 0.06	3.41 ± 1.15	5.26 ± 1.76	4.53 ± 1.17	5.99 ± 1.80	6.71 ± 1.93	1.765	0.195

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

Table 5
qPCR primers.

Target gene	Reference	Forward (5′–3′)	Reverse(5′–3′)
TLR22[a]	Xiao et al. (2010)	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.

$$\text{Survival rate} = \frac{N_{ft}}{N_{fi}} \times 100$$

$$\text{Cumulative mortality rate} = \frac{(N_0 - N - N_t)}{N_0} \times 100$$

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 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).

3. Results

3.1. Survival and growth

The survival rate increased from 93.6% to 96.9% with dietary n-3 HUFA increasing from 0.15% to 0.98%, and thereafter decreased. Fish fed diets with 2.25% n-3 HUFA had the lowest survival rate (91.1%), but no significant differences were observed in survival rate among dietary treatments ($P > 0.05$) (Table 3). The specific growth rate (SGR) and feed intake (FI) showed a similar trend with the survival rate as dietary n-3 HUFA increased. SGR of fish in the control group with 0.15% HUFA (1.82% day^{-1}) was the lowest, significantly lower than that of fish fed the diet with 0.98% dietary n-3 HUFA (2.24% day^{-1}). There were no significant differences in SGR of fish fed diets with equal to or exceeding 0.60% dietary n-3 HUFA ($P > 0.05$) (Table 3). When n-3 HUFA concentration increased from 0.15% to 0.98%, FI significantly increased from 1126.7 g to 1542.8 g, and thereafter decreased to 1315.5 g with dietary n-3 HUFA increased to 2.25% ($P < 0.05$). FI in the control group (0.15% n-3 HUFA) was significantly lower than those in the low and moderate n-3 HUFA groups (0.60%, 0.98% and 1.37% n-3 HUFA) ($P < 0.05$) and comparable to those in the high n-3 HUFA treatments (1.79% and 2.25%).

3.2. Immunological parameters

The NBT positive cell percent and serum SOD activity significantly increased with increasing dietary n-3 HUFA from 0.15% to 0.60% ($P < 0.05$) and then decreased as dietary n-3 HUFA increased to 2.25%. The NBT positive cell percent was lowest in the 0.15% n-3 HUFA group (46.61%) significantly lower than that in the 0.60% n-3 HUFA group (75.04%) but comparable to those in fish fed diets with equal to or higher than 0.98% n-3 HUFA (55.46–61.75%). No significance was found in NBT positive cell percent among fish fed the diets with equal to or higher than 0.98% n-3 HUFA ($P > 0.05$). Serum SOD activity markedly increased from 73.54 to 99.46 units ml^{-1} with dietary n-3 HUFA increased from 0.15% to 0.60%, and thereafter significantly decreased to 75.41 units ml^{-1} as n-3 HUFA increased from 0.60% to 1.79% ($P < 0.05$). No statistics differences were detected in serum SOD activity among fish fed the diets with equal to or higher than 0.98% n-3 HUFA. The lysozyme activity significantly increased with increasing dietary n-3 HUFA from 0.15% to 1.37% ($P < 0.05$), and then decreased. Fish fed the diets with 1.37% dietary n-3 HUFA had significantly higher lysozyme activity in serum than those fed the diets containing n-3 HUFA equal to or below 0.60% and no significant differences were observed in serum lysozyme activity among fish fed diets with equal to or exceeding 0.98% dietary n-3 HUFA. There were no significant differences in PI among dietary groups ($P > 0.05$) (Table 4 and 6–8).

3.3. Cumulative mortality rate

The cumulative mortality significantly decreased from 67.9% to 46.7% with increasing dietary n-3 HUFA from 0.15% to 0.60%, and then significantly increased to 76.9% with further increase of n-3 HUFA from 0.60% to 2.25% ($P < 0.05$). Fish fed diets with 0.60% n-3 HUFA had significantly lower cumulative mortality (46.7%) than other groups except the 0.98% level group (61.6%). No significant differences were observed in the cumulative mortality between the control (0.15% HUFA) and the groups with dietary n-3 HUFA equal to or above 0.98% ($P > 0.05$) (Fig. 1).

3.4. Immune related gene expression

Relative mRNA expression of TLR22 and MyD88 at the early stage after natural infestation of parasites, *C. irritans* in the kidney (a, b) and liver (c, d) of large yellow croaker, *L. crocea* fed with graded levels of dietary n-3 HUFA were presented in Fig. 2. In kidney, there were no significant differences in the expression levels of TLR22 among dietary treatments ($P > 0.05$) (Fig. 2.a). MyD88 mRNA levels significantly increased to the maximum levels in the group with 0.98% n-3 HUFA, and thereafter significantly dropped when dietary n-3 HUFA increased to 2.25%. The expression levels of MyD88 transcript were increased by approximately 1.06-fold, 0.79-fold, 0.35-fold and 0.36-fold in treatments with 0.98%, 1.37%, 1.79% and 2.25% dietary n-3 HUFA, respectively ($P < 0.05$) (Fig. 2b). In liver, TLR22 expression levels significantly increased to the maximum levels when dietary n-3 HUFA increased from 0.15% to 0.98%, and then decreased with n-3 HUFA increased to 2.25%. The mRNA expression levels of TLR22 were increased by about 1.53-fold, 0.91-fold and 1.14-fold in treatments with 0.98%, 1.37% and 1.79% dietary n-3 HUFA, respectively ($P < 0.05$) (Fig. 2c). The expression levels of MyD88 transcript were up-regulated to the maximum (0.37-fold higher than the control group) and down-regulated to the minimum (0.25-fold lower than the control group) in treatments with 0.98% and 2.25% dietary n-3 HUFA, respectively ($P < 0.05$) (Fig. 2d).

Relative mRNA expression of TLR22 and MyD88 at the later stage after natural infestation of parasites, *C. irritans* in the kidney (a, b) and liver (c, d) of large yellow croaker, *L. crocea* fed with graded levels of dietary n-3 HUFA were presented in Fig. 3. In kidney, the expression levels of TLR22 transcript significantly increased as dietary n-3 HUFA increased from 0.15% to 0.60%, and then decreased with increasing dietary n-3 HUFA from 0.60% to 2.25% ($P < 0.05$). TLR22 transcript levels were increased by

Table 6
Fatty acid composition (% total fatty acids) in the carcass of juvenile large yellow croaker fed the diets with graded levels of n-3 HUFA.[a]

Fatty acid	Dietary n-3 HUFA contents (% dry weight)					
	0.15	0.60	0.98	1.37	1.79	2.25
14:0	3.88 ± 0.30	3.29 ± 0.92	3.63 ± 0.28	3.19 ± 0.15	2.92 ± 0.21	4.13 ± 0.19
16:0	40.40 ± 0.16a	42.50 ± 1.17a	42.73 ± 1.45a	39.44 ± 0.75a	33.70 ± 0.60b	31.57 ± 1.53b
18:0	7.15 ± 0.30ab	8.13 ± 0.15a	6.52 ± 0.28b	6.63 ± 0.36b	6.05 ± 0.23bc	4.90 ± 0.07c
20:0	1.90 ± 0.16	1.34 ± 0.27	1.89 ± 0.20	1.99 ± 0.08	1.37 ± 0.03	1.62 ± 0.22
∑SFA[b]	53.33 ± 0.25a	54.63 ± 1.63a	54.77 ± 1.37a	51.37 ± 0.90a	44.04 ± 0.59b	42.21 ± 1.20b
16:1	7.80 ± 0.05a	7.31 ± 0.22ab	6.66 ± 0.18b	4.86 ± 0.24c	4.19 ± 0.05c	4.57 ± 0.11c
18:1	21.46 ± 0.31a	19.34 ± 0.37b	18.03 ± 0.25c	16.23 ± 0.14d	15.69 ± 0.15d	14.00 ± 0.07e
∑MUFA[c]	29.26 ± 0.27a	26.64 ± 0.24b	24.70 ± 0.10c	21.09 ± 0.36d	19.72 ± 0.05e	18.57 ± 0.04e
18:2n-6	8.60 ± 0.11	9.91 ± 1.13	9.60 ± 0.41	10.16 ± 0.41	9.95 ± 0.41	10.71 ± 1.42
20:4n-6	1.69 ± 0.15a	1.89 ± 0.16a	2.15 ± 0.04ab	2.73 ± 0.15bc	3.10 ± 0.09c	3.35 ± 0.18c
∑n-6 PUFA[d]	10.29 ± 0.18a	11.80 ± 1.29a	11.75 ± 0.42a	12.89 ± 0.53ab	13.05 ± 0.50ab	15.66 ± 0.09b
18:3n-3	0.68 ± 0.01a	0.87 ± 0.05b	0.92 ± 0.01b	0.96 ± 0.03bc	1.10 ± 0.03cd	1.20 ± 0.05d
20:5n-3	0.83 ± 0.07a	0.94 ± 0.01a	1.73 ± 0.05b	2.49 ± 0.08c	4.04 ± 0.16d	4.64 ± 0.07e
22:6n-3	1.84 ± 0.15a	1.84 ± 0.08a	3.53 ± 0.26b	5.92 ± 0.18c	9.66 ± 0.19d	9.91 ± 0.05d
∑n-3 PUFA[e]	3.53 ± 0.38a	3.36 ± 0.20a	6.17 ± 0.30b	9.36 ± 0.29c	14.80 ± 0.32d	16.21 ± 0.60d
n-3/n-6PUFA	0.34 ± 0.03ab	0.29 ± 0.02a	0.53 ± 0.04bc	0.73 ± 0.05c	1.14 ± 0.07d	1.01 ± 0.01d
n-3HUFA[f]	2.67 ± 0.19a	2.78 ± 0.09a	5.26 ± 0.30b	8.41 ± 0.25c	13.70 ± 0.30d	15.02 ± 0.52d
ARA/EPA[g]	2.05 ± 0.01a	2.01 ± 0.18a	1.25 ± 0.03b	1.10 ± 0.09bc	0.77 ± 0.05c	0.72 ± 0.05c
DHA/EPA[h]	2.24 ± 0.10ab	1.96 ± 0.07a	2.04 ± 0.09ab	2.38 ± 0.00b	2.40 ± 0.08b	2.23 ± 0.07ab

[a] Values are means ± S.E.M. of three replicate cages. Different letters in each row show significant differences among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: standard error of means.
[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.

approximately 1.17-fold, 0.76-fold and 0.65-fold in treatments with 0.60%, 0.98% and 1.37% dietary n-3 HUFA, respectively ($P < 0.05$) (Fig. 3a). However, no significant differences were found in the expression levels of MyD88 among dietary treatments ($P > 0.05$) (Fig. 3b). In liver, the expression levels of TLR22 transcript were increased by approximately 0.79-fold, 0.79-fold and 0.88-fold in treatments with 0.60%, 0.98% and 1.37% dietary n-3 HUFA, respectively ($P < 0.05$). Thereafter, TLR22 transcriptional levels decreased as dietary n-3 HUFA increased to 2.25% ($P > 0.05$) (Fig. 3c). MyD88 transcript levels increased with increasing dietary n-3 HUFA from 0.15% to 1.37%, and then decreased as dietary n-3 HUFA increased to 2.25%. The expression levels of MyD88 transcript were up-

Table 7
Fatty acid composition (% total fatty acids) in the muscle of juvenile large yellow croaker fed the diets with graded levels of n-3 HUFA.[a]

Fatty acid	Dietary n-3 HUFA contents (% dry weight)					
	0.15	0.60	0.98	1.37	1.79	2.25
14:0	3.35 ± 0.30a	2.20 ± 0.15b	2.60 ± 0.25ab	2.73 ± 0.07ab	3.06 ± 0.11ab	3.19 ± 0.18a
16:0	28.39 ± 0.48ac	36.52 ± 0.80b	32.03 ± 1.52c	30.93 ± 0.57c	29.78 ± 0.84ac	26.72 ± 0.56a
18:0	6.42 ± 0.34ab	7.19 ± 0.51a	6.29 ± 0.43ab	5.11 ± 0.22b	4.93 ± 0.05b	5.00 ± 0.15b
20:0	2.51 ± 0.23a	1.54 ± 0.01b	1.96 ± 0.25ab	1.82 ± 0.02ab	1.89 ± 0.09ab	1.63 ± 0.21b
∑SFA[b]	40.67 ± 0.37a	47.44 ± 0.39b	42.88 ± 1.38a	40.59 ± 0.82a	39.66 ± 0.98ac	36.55 ± 0.42c
16:1	6.47 ± 0.30a	5.12 ± 0.33ab	4.36 ± 0.40bc	4.07 ± 0.31bc	3.85 ± 0.22bc	3.42 ± 0.32c
18:1	20.52 ± 0.27a	17.18 ± 0.89ab	15.36 ± 0.96bc	14.77 ± 0.55bc	13.65 ± 0.98bc	13.32 ± 0.87c
∑MUFA[c]	26.99 ± 0.24a	22.30 ± 1.12ab	19.71 ± 1.36bc	18.85 ± 0.86bc	17.50 ± 1.20bc	16.74 ± 1.19c
18:2n-6	7.49 ± 0.20a	12.40 ± 0.14b	12.13 ± 1.22b	10.52 ± 0.48b	10.59 ± 0.60b	10.92 ± 0.36b
20:4n-6	3.09 ± 0.06	3.58 ± 0.09	3.37 ± 0.26	3.40 ± 0.05	3.57 ± 0.10	3.46 ± 0.14
∑n-6 PUFA[d]	10.52 ± 0.21a	15.99 ± 0.17b	15.70 ± 1.53b	13.92 ± 0.60b	14.16 ± 0.56b	14.38 ± 0.41b
18:3n-3	1.12 ± 0.07a	1.47 ± 0.04b	1.36 ± 0.12ab	1.31 ± 0.04ab	1.39 ± 0.06ab	1.33 ± 0.04ab
20:5n-3	3.01 ± 0.31a	2.66 ± 0.15a	3.50 ± 0.20ab	4.47 ± 0.17bc	5.03 ± 0.21cd	5.71 ± 0.21d
22:6n-3	6.28 ± 0.83ab	6.10 ± 0.42a	9.06 ± 0.61bc	10.97 ± 0.58bcd	11.33 ± 0.16cd	13.60 ± 0.68d
∑n-3 PUFA[e]	10.41 ± 1.06ab	10.22 ± 0.62a	13.92 ± 0.78bc	16.76 ± 0.72cd	17.76 ± 0.42de	20.64 ± 0.90e
n-3/n-6PUFA	1.28 ± 0.01a	0.64 ± 0.04b	0.85 ± 0.07b	1.21 ± 0.08a	1.37 ± 0.03a	1.44 ± 0.06a
n-3HUFA[f]	9.29 ± 1.12ab	8.75 ± 0.57a	12.56 ± 0.81bc	15.45 ± 0.74cd	16.36 ± 0.37de	19.31 ± 0.88e
ARA/EPA[g]	1.06 ± 0.14ab	1.36 ± 0.06b	0.97 ± 0.11ac	0.76 ± 0.02ac	0.71 ± 0.01ac	0.61 ± 0.01c
DHA/EPA[h]	2.75 ± 0.07a	2.29 ± 0.03c	2.58 ± 0.04ab	2.45 ± 0.05bc	2.26 ± 0.07c	2.38 ± 0.03bc

[a] Values are means ± S.E.M. of three replicate cages. Different letters in each row show significant differences among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: standard error of means.
[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.

Table 8
Fatty acid composition (% total fatty acids) in the liver of juvenile large yellow croaker fed the diets with graded levels of n-3 HUFA.[a]

Fatty acid	Dietary n-3 HUFA contents (% dry weight)					
	0.15	0.60	0.98	1.37	1.79	2.25
14:0	1.25 ± 0.01a	1.22 ± 0.03a	1.55 ± 0.55ab	1.90 ± 0.09b	2.42 ± 0.06c	2.65 ± 0.18c
16:0	34.80 ± 0.74b	35.79 ± 0.46b	39.93 ± 1.49a	35.55 ± 0.15b	31.04 ± 0.09c	27.14 ± 0.28d
18:0	9.31 ± 0.28a	9.33 ± 0.29a	6.80 ± 0.85b	5.66 ± 0.64b	4.52 ± 0.14b	5.50 ± 0.36b
20:0	1.08 ± 0.07a	0.97 ± 0.02ab	0.91 ± 0.07ab	0.84 ± 0.05b	0.95 ± 0.02ab	1.03 ± 0.04ab
∑SFA[b]	46.44 ± 0.42b	47.31 ± 0.23ab	49.19 ± 0.78a	43.95 ± 0.49c	38.92 ± 0.21d	36.31 ± 0.23e
16:1	7.37 ± 0.19a	5.99 ± 0.42ab	5.01 ± 0.20abc	4.97 ± 0.82abc	3.93 ± 0.78bc	3.31 ± 0.31c
18:1	19.56 ± 1.04a	17.70 ± 0.61a	14.01 ± 0.19b	12.60 ± 0.35b	11.42 ± 0.50b	12.76 ± 1.07b
∑MUFA[c]	26.92 ± 1.22a	23.69 ± 1.03ab	19.02 ± 0.21bc	17.57 ± 0.48c	15.36 ± 1.28c	16.06 ± 1.37c
18:2n-6	16.47 ± 0.30	16.04 ± 0.93	15.76 ± 0.51	15.73 ± 0.08	16.33 ± 0.49	15.26 ± 0.61
20:4n-6	1.99 ± 0.01a	3.04 ± 0.01b	4.04 ± 0.05c	3.64 ± 0.06cd	4.19 ± 0.15d	4.01 ± 0.15cd
∑n-6 PUFA[d]	17.80 ± 0.96	19.19 ± 0.93	19.80 ± 0.55	19.37 ± 0.05	20.53 ± 0.64	19.26 ± 0.73
18:3n-3	0.97 ± 0.14a	1.34 ± 0.11ab	1.14 ± 0.03ab	1.39 ± 0.02b	1.44 ± 0.02b	1.41 ± 0.08b
20:5n-3	0.28 ± 0.22a	0.94 ± 0.04ab	1.54 ± 0.07bc	2.42 ± 0.11c	3.65 ± 0.10d	4.41 ± 0.37d
22:6n-3	0.83 ± 0.03a	1.91 ± 0.10a	4.64 ± 0.15b	5.95 ± 0.16b	9.34 ± 0.54c	11.53 ± 0.62d
∑n-3 PUFA[e]	2.00 ± 0.19a	4.19 ± 0.06b	7.33 ± 0.25c	9.77 ± 0.25d	14.43 ± 0.42e	17.34 ± 0.76f
n-3/n-6PUFA	0.11 ± 0.01a	0.22 ± 0.01b	0.37 ± 0.02c	0.50 ± 0.01d	0.98 ± 0.00e	0.90 ± 0.02f
n-3HUFA[f]	1.11 ± 0.05a	2.85 ± 0.05b	6.19 ± 0.22c	8.37 ± 0.26d	12.99 ± 0.44e	15.93 ± 0.69f
ARA/EPA[g]	7.70 ± 0.10a	3.36 ± 0.17b	2.64 ± 0.14c	1.51 ± 0.05d	1.22 ± 0.01d	0.97 ± 0.11d
DHA/EPA[h]	2.14 ± 1.07	2.05 ± 0.21	3.02 ± 0.05	2.47 ± 0.06	2.57 ± 0.22	2.66 ± 0.29

[a] Values are means ± S.E.M. of three replicate cages. Different letters in each row show significant differences among dietary treatments by Tukey's test ($P < 0.05$). S.E.M.: standard error of means.
[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.

regulated to the maximum (0.25-fold higher than the control group) in treatments with 1.37% dietary n-3 HUFA ($P > 0.05$). The lowest expression levels of MyD88 were found in treatments with 1.79% and 2.25% dietary n-3 HUFA (0.40-fold lower than the 1.37% n-3 HUFA group) (Fig. 3d).

4. Discussion

Results of the present study showed that juvenile large yellow croaker fed the diets containing 0.60% and 0.98% n-3 HUFA had the best immunity and growth, respectively. The optimum dietary n-3 HUFA level (0.60–0.98% d.w.) is similar to the recommended values (0.5–1.0% d.w.) reported by previous studies [3,4,33–36], but lower than those (1.3–2.5% d.w.) in other studies [5,37,38]. The differences could be due to species, DHA/EPA ratio or dietary lipid levels. Requirement of n-3 HUFA has been found to decrease with the increase of dietary DHA/EPA in the same fish species [39], indicating DHA has a relatively higher EFA value than EPA [40]. Glencross [41] found EFA requirement was positively correlated with dietary lipid level and suggested EFA should be defined in conjunction with dietary lipid levels. Thus, in the present study, n-3 HUFA requirement (0.60–0.98% d.w.) should be considered with a DHA/EPA (around 2.0), and a diet containing 11% crude lipid.

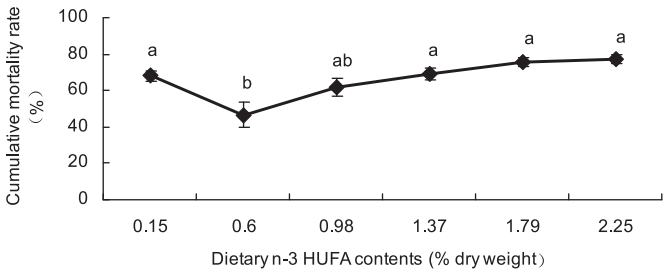

Fig. 1. The 13 d cumulative mortality rate of juvenile large yellow croaker among different dietary group during natural infestation of parasites, *Cryptocaryon irritans*. Values are means ± S.E.M. ($n = 3$). Different letters indicate significant difference in cumulative mortality among dietary groups by Tukey's test ($P < 0.05$).

NBT positive cell percentage is the percentage of leucocytes with dark blue formazan and thus can be seen as a capacity of leucocytes to produce intracellular reactive oxygen species (ROS). Serum lysozyme activity, SOD activity and NBT positive cell percentage were significantly higher in the low or moderate n-3 HUFA treatment compared with the control group, indicating the enhancement of fish immune function by dietary n-3 HUFA. 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 [42–44]. A previous study found that phagocytic function was enhanced by appropriate supplementation of dietary n-3 HUFA [45]. However, PI showed no significant differences among dietary treatments in the present study, which may be due to immune fatigue caused by parasite infection. This was confirmed by the findings of Sitjà-Bobadilla et al. [46] who also found that total serum peroxidases and lysozyme activity of gilthead sea bream could be initially increased in response to parasite exposure, but quickly exhausted to fight it, and did not recover even in the uninfected fish.

After bacteria are ingested by phagocytes, ROS exert a critical role in bacterial killing [47,48] 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 [49]. In addition, a genetic defect in TLR4 could be responsible for the impairment of neutrophils in generating superoxide anion in response to LPS [50] and ROS production after vascular injury was attenuated in TLR2 knockout mice compared with control littermates [51]. Herein, it was found that intracellular ROS production, reflected by the parameter of NBT positive cell percentage, seemed to be positively correlated with the relative TLR22 and MyD88 mRNA expression with significantly higher

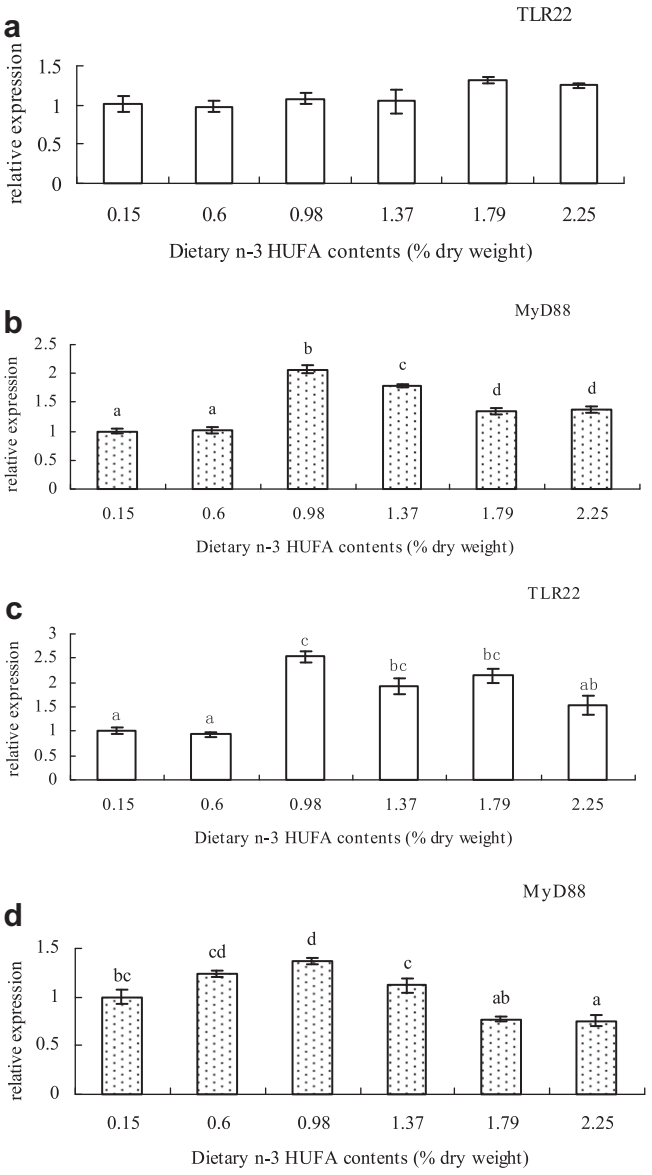

Fig. 2. Relative mRNA expression of TLR22 and MyD88 at the early stage after natural infestation of parasites, *Cryptocaryon irritans* in the kidney (a, b) and liver (c, d) of large yellow croaker, *Larmichthys crocea* fed with graded levels of dietary n-3 HUFA. Values are means ± S.E.M. ($n = 3$). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

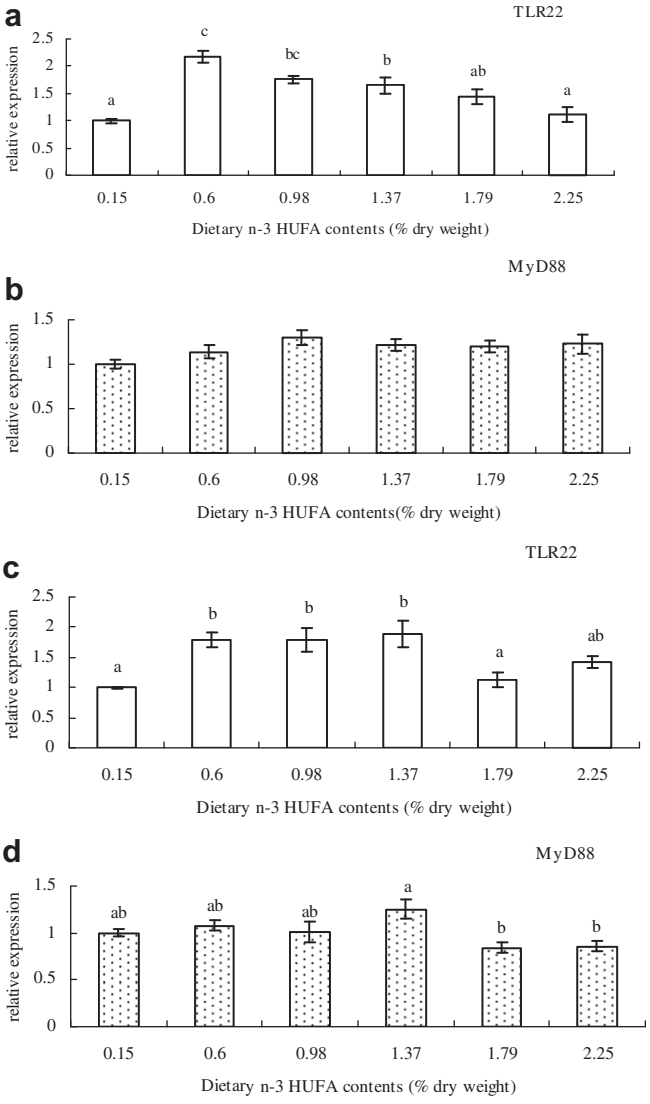

Fig. 3. Relative mRNA expression of TLR22 and MyD88 at the later stage after natural infestation of parasites, *Cryptocaryon irritans* in the kidney (a, b) and liver (c, d) of large yellow croaker, *Larmichthys crocea* fed with graded levels of dietary n-3 HUFA. Values are means ± S.E.M. ($n = 3$). Bars bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

mRNA expression observed in the low or moderate n-3 HUFA treatment. Although there is scarce information on the effects of dietary n-3 HUFA on the expression of TLRs and MyD88, one supporting evidence was from Gabler et al. [52] who found TLR4 expression was increased in the adipose tissues of pigs fed diets with 7.5% fish oil of dry diet compared to the n-3 HUFA deprived group after LPS injection.

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 [53–56]. ROS have been proved to be involved in some other diseases such as cancers, diabetes mellitus, atherosclerosis etc [57] where inflammatory cytokines exert an important role and diets rich on n-3 PUFA have been reported to have clear anti-inflammatory effects [58]. Thus, high inclusion of n-3 HUFA may protect fish from cell and tissue damage through inhibiting the overproduction of ROS, which may be accomplished by a down-regulation of TLR22 and MyD88 as described in the present study. It has been reported that anti-inflammatory effects of the n-3 PUFA may require direct interactions with the TLRs receptor [59]. However, in the present study, a downregulation of TLR22 and MyD88 and significantly lower ROS production was observed in high dietary n-3 HUFA treatment, indicating direct interaction may be not the only pathway to exert anti-inflammatory effects of n-3 HUFA. Though it is uncertain how n-3 HUFA regulates the expression of TLR22 and MyD88, there are some studies which have shown direct and indirect modulation of TLR signaling by members of the suppressors of cytokine signaling (SOCS) family [48,60,61].

The 13 d cumulative mortality during the natural infestation of parasites was significantly decreased in fish fed diets with relatively low n-3 HUFA (0.60%) in large yellow croaker, which could be attributed to the enhanced nonspecific immunity modulated by TLR22 and MyD88. Previous studies have shown that TLR2 and TLR4 mRNA expression was up-regulated in cecum and spleen in response to *Salmonella enteritidis* infection in young chicks [62] and

MyD88-deficient mice are more susceptible to many different microbial pathogens [63–66], suggesting the important role of TLRs and MyD88 in the disease recognition and defenses.

In summary, results of the present study showed that low or moderate levels of n-3 HUFA could significantly improve growth performance, nonspecific immunity, protection against parasite infection, as well as hepatic and kidney mRNA expression of TLR22 and MyD88 in juvenile large yellow croaker. Further information is required to determine the consequences of dietary n-3 HUFA on other components of TLR signaling pathway, such as proinflammatory cytokines, at mRNA and protein levels to understand how dietary n-3 HUFA modulate the immune system of marine fish.

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