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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.