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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.