Dietary lipid requirement of grouper, *Epinephelus malabaricus*, and effects on immune responses

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**Abstract**

A growth trial was conducted to determine the dietary lipid requirement and its effects on immune responses of juvenile grouper. Purified diets with five dietary lipid levels (0%, 4%, 8%, 12% and 16%; fish oil/corn oil = 1:1) were each fed to triplicate groups of grouper (mean initial weight: 4.43 ± 0.07 g) in a recirculating rearing system maintained at 29 ± 1 °C for 8 weeks. Weight gain was highest (*P* < 0.05) in fish fed diets with 4–12% lipid, followed by the 16% lipid group, and lowest in fish fed the lipid-free control diet. Feed efficiency (FE) and protein efficiency ratio (PER) in fish followed the same pattern of weight gain. Body lipid content was highest in fish fed with 12% and 16% lipid diets, followed by 4% and 8% lipid groups, and lowest in fish fed the control diet. Fish fed with the control diet had lower survival than the other dietary groups. Fish fed with lipid-containing diets had higher white blood cell (WBC) count and respiratory burst activity of leukocyte than fish fed with the control diet. Plasma lysozyme activity of fish fed with 12% and 16% lipid diets were higher than for fish fed with ≤ 4% lipid diets. Alternative complement activity (ACH50) was higher in fish-fed diets with ≥ 8% lipid than fish fed with the control diet. Analysis by second-order regression of weight gain indicated that the optimal dietary lipid level in juvenile grouper is about 9%. Four percent of dietary lipid appears to meet the minimal requirement for grouper. These data also suggest that dietary lipid supplementation enhances the immune response of grouper.

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**Keywords:** Lipid requirement; Immune response; Grouper

1. **Introduction**

Grouper are high quality seafood in Asia and around the world. They are also good candidates for intensive aquaculture because of their desirable taste, hardiness in a
crowded environment and rapid growth. However, information about nutrient requirements of grouper are scarce. The dietary protein requirement (Chen and Tsai, 1994; Shiau and Lan, 1996) and carbohydrate utilization (Shiau and Lin, 2001) for Epinephelus malabaricus have been investigated. Recently, Wu et al. (2002) reported that docosahexaenoic acid (DHA) was superior to eicosapentaenoic acid (EPA) as an essential fatty acid for growth of grouper. However, the dietary lipid requirement of grouper is not known.

The immune system can be influenced by a wide range of factors, including disease, pollutants, hormones and diet (Pulsford et al., 1995). Recent evidence suggests that nutritional status and immune status are tightly linked (Landolt, 1989; Lygren and Waagbo, 1999). Lipid is an important component of the diet, both as an energy source and as the source of essential fatty acids, which fish cannot synthesize but need for basic functions, including growth and maintenance of healthy tissues (Watanabe, 1982). Furthermore, both the concentration and type of dietary fats have been reported to alter various indices of immune response in human (Kelley and Daudu, 1993) and several animal studies (Boissonneault and Johnston, 1983; Korver et al., 1997). However, the effects of lipid on immune response in fish is not clear.

The purpose of this study was to estimate the lipid requirement and its effects on immune response of grouper. White blood cell (WBC) count, respiratory burst activity of leukocyte (O2 production), lysozyme and alternative complement (ACH50) activity were used as indicators of immunity.

2. Materials and methods

2.1. Diet preparation

Diets were prepared with five lipid levels as 0%, 4%, 8%, 12% and 16%. The lipid sources were equally supplemented by fish oil (Scott and Bowne, London, UK) and corn oil (Tai-Tang Industrial, Taiwan). Casein (Sigma, St. Louis, MO) and corn starch (Sigma) were used as dietary protein and carbohydrate sources, respectively. The diets were kept isoenergetic by adjusting the corn starch and cellulose content in the diet. An attractant that had a similar chemical composition to squid mantle tissue (Mackie and Mitchell, 1985) was added at 6% to all diets to increase palatability and diet acceptance. The experimental diet formulation and proximate analysis (Association of Official Analytical Chemists, 1995) are given in Table 1. Diet preparation was according to Shiau and Lin (2001). All diets were stored at −20 °C until fed.

2.2. Experimental procedure

Epinephelus malabaricus juveniles were obtained from a local fish fry dealer. Upon arrival, they were acclimatized to laboratory conditions for 4 weeks in a 1,000-l plastic tank and fed with a commercial diet (Lucky Star; Hung Kuo Industrial, Taipei, Taiwan). The proximate composition (%) of the commercial diet was: moisture, 11.7; crude protein,
43.3; lipid, 8.8; ash, 9.3. At the beginning of experiment, 12 fish (mean weight, 4.43 ± 0.07 g) were stocked in each aquarium (30.5 × 61.0 × 55.5 cm). Each experimental diet was fed to fish in three aquaria. Diets were randomly assigned to groups of fish. Each aquarium was part of a closed recirculating system with a common reservoir of water at 29–32‰ salinity. The water was circulated at 2 l/min through two separate biofilters to remove impurities and reduce ammonia concentrations. A photoperiod of 12 h light (0800–2000 h), 12 h dark was used.

The fish were fed with 3% of their body weight per day. This amount was close to the maximal daily ration for grouper according to feed consumption during the acclimation period. The daily ration was divided into two equal meals fed at 0830 and 1630 h. Fish were weighed once every 2 weeks and the daily ration adjusted accordingly. Any dead fish were removed and were not replaced during the experiment. The fish were fed with the test diets for an 8-week period.

At the end of the feeding trial, the fish were bulk weighed from each aquarium. Weight gain (as measured by the percentage of body weight gain in each
aquarium)[100 \times \text{[final body weight-initial body weight]}/\text{initial body weight}], feed efficiency (FE)[\text{[final body weight-initial body weight]}/\text{feed intake}] and protein efficiency ratio (PER)[\text{[final body weight-initial body weight]}/\text{protein intake}] were calculated. After the final weighing, three fish were randomly sampled from each aquarium and pooled for body composition analysis (Association of Official Analytical Chemists, 1995). Blood was collected from the caudal vein by the syringe with heparin as the anticoagulant from four fish of each aquarium and pooled for white blood cell (WBC) count, measured by automated hematology analyzer (KX-21, Sysmex, Japan).

Leukocytes were isolated from the blood of grouper by the lymphoprep medium (density: 1.077; Gibco BRL, Life Technologies). The isolated leukocytes were cultured in Aim V/Leibovitz’s L15 medium (Gibco BRL) containing 5.5 mM glucose (Sigma). Respiratory burst activity [intracellular superoxide anion (O$_2^-$) production ratio] of leukocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production, as described by Secombes (1990).

Remaining blood was centrifuged at 3,000 \times g for 15 min. Plasma was removed and stored at \(-80^\circ\text{C}\). Alternative complement pathway (ACH50) activity was estimated, as described by Montero et al. (1998) and the ACH50 units were defined as the concentration of plasma giving 50% hemolysis of sheep red blood cells. The turbidimetric assay for lysozyme activity was carried out according to Obach et al. (1993). All analysis were conducted in triplicate.

2.3. Statistical analysis

Each experimental diet was fed to three groups of fish according to a completely randomized design. Results were analyzed by one-way analysis of variance (ANOVA) with five lipid levels as main effect, using the SAS/PC statistical software, and significance was set at \(P<0.05\). Multiple comparisons among means were made with Duncan’s new multiple range test. Dietary lipid requirement for juvenile

### Table 2

Weight gain, feed efficiency (FE), protein efficiency ratio (PER) and survival of grouper fed different levels of lipid diets

<table>
<thead>
<tr>
<th>Dietary lipid level (%)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (%)</td>
<td>71.41±21.76$^c$</td>
<td>442.87±39.54$^a$</td>
<td>393.73±20.73$^a$</td>
<td>384.34±31.11$^a$</td>
<td>277.15±29.74$^b$</td>
</tr>
<tr>
<td>FE$^2$</td>
<td>0.20±0.05$^c$</td>
<td>0.84±0.04$^a$</td>
<td>0.78±0.03$^a$</td>
<td>0.78±0.05$^a$</td>
<td>0.63±0.04$^b$</td>
</tr>
<tr>
<td>PER$^3$</td>
<td>0.40±0.10$^c$</td>
<td>1.67±0.08$^a$</td>
<td>1.56±0.06$^a$</td>
<td>1.55±0.10$^a$</td>
<td>1.25±0.08$^b$</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>61.11±17.35$^b$</td>
<td>86.11±17.35$^a$</td>
<td>97.22±4.81$^a$</td>
<td>91.67±14.43$^a$</td>
<td>100.0±0.00$^a$</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant \((P<0.05)\) difference between different dietary lipid level.

1 Values are means ± SD from three groups of fish \((n=3)\).
2 g weight gain/g feed consumed.
3 g weight gain/g protein consumed.
grouper was estimated by the polynomial regression based on weight gain of the fish.

3. Results

Weight gain, feed efficiency (FE), protein efficiency ratio (PER) and survival of grouper fed different diets are presented in Table 2. Weight gain were highest ($P < 0.05$) in grouper-fed diets with 4–12% lipid, followed by 16% lipid group and lowest in fish fed with the lipid-free control diet. Feed efficiency and PER in fish followed the same pattern of weight gain. Fish fed with the control diet had lower survival than all the other dietary treatments.

Fish fed diets with $\geq$ 12% lipid had lower body moisture content than fish fed with 8% lipid and the control diet (Table 3). Fish fed with the control diet had highest ash content, followed by 4% lipid group, and lowest in fish fed with $\geq$ 8% lipid diets. Body lipid contents were highest in fish fed with $\geq$ 12% lipid diets, followed by 4% and 8% lipid groups, and the lowest in fish fed with the control diet. Body protein content in grouper was not affected by the dietary treatments.

White blood cell (WBC) count and respiratory burst activity of leukocytes of fish fed with lipid-containing diets were higher than those of fish fed with the control diet (Table 4). Plasma lysozyme activity of fish fed with 12% and 16% lipid diets were higher than fish fed with $\leq$ 4% lipid diets. Alternative complement activity

<table>
<thead>
<tr>
<th>Dietary lipid level (%)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>74.97 ± 0.68$^a$</td>
<td>73.49 ± 0.83$^b$</td>
<td>74.83 ± 0.93$^a$</td>
<td>73.41 ± 0.12$^b$</td>
<td>73.40 ± 0.34$^b$</td>
</tr>
<tr>
<td>Ash</td>
<td>5.99 ± 0.65$^a$</td>
<td>4.13 ± 0.54$^b$</td>
<td>2.95 ± 0.39$^c$</td>
<td>2.77 ± 0.32$^b$</td>
<td>2.99 ± 0.20$^c$</td>
</tr>
<tr>
<td>Crude protein</td>
<td>16.12 ± 0.87</td>
<td>16.44 ± 1.01</td>
<td>15.98 ± 0.79</td>
<td>16.15 ± 0.69</td>
<td>16.46 ± 1.05</td>
</tr>
<tr>
<td>Ether extract</td>
<td>1.44 ± 0.18$^c$</td>
<td>3.72 ± 0.42$^b$</td>
<td>4.12 ± 0.31$^b$</td>
<td>5.71 ± 0.37$^a$</td>
<td>6.39 ± 0.83$^a$</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant ($P < 0.05$) difference between different dietary lipid level.

1 Values are means ± SD from three groups of fish ($n = 3$) with three fish per group.

Table 4

<table>
<thead>
<tr>
<th>Dietary lipid level (%)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (cell/ml)</td>
<td>59.7 ± 2.9$^b$</td>
<td>73.5 ± 8.7$^a$</td>
<td>72.4 ± 3.6$^a$</td>
<td>82.9 ± 14.6$^a$</td>
<td>74.6 ± 13.6$^a$</td>
</tr>
<tr>
<td>O$_2$ production ratio</td>
<td>0.87 ± 0.15$^b$</td>
<td>1.41 ± 0.20$^a$</td>
<td>1.55 ± 0.19$^a$</td>
<td>1.63 ± 0.18$^a$</td>
<td>1.50 ± 0.28$^a$</td>
</tr>
<tr>
<td>Lysozyme (U/ml)</td>
<td>1.54 ± 0.1$^c$</td>
<td>2.66 ± 0.99$^c$</td>
<td>2.90 ± 0.74$^{bc}$</td>
<td>4.96 ± 0.75$^{ab}$</td>
<td>5.13 ± 2.15$^a$</td>
</tr>
<tr>
<td>ACH50 (U/ml)</td>
<td>91.12 ± 5.88$^b$</td>
<td>103.22 ± 10.23$^{ab}$</td>
<td>122.32 ± 9.66$^a$</td>
<td>124.12 ± 10.14$^a$</td>
<td>129.86 ± 12.70$^a$</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant ($P < 0.05$) difference between different dietary lipid level.

1 Values are means ± SD from three groups of fish ($n = 3$) with four fish per group.
(ACH50) was higher in fish fed diets with $\geq 8\%$ lipid than fish fed with the control diet.

When the second-order regression was employed, based on weight gain for estimating the dietary lipid requirement of *E. malabaricus* (as shown in Fig. 1), the regression equation was as follows: $Y = -4.54X^2 + 79.01X + 107.95$. The optimum dietary lipid requirement for juvenile grouper is estimated to be 8.7\%.

### 4. Discussion

Both broken-line and regression methods have generally been used to quantify nutrient requirements in aquatic species (Shiau, 2001). The broken-line technique involves using two straight lines to model the dose–response relationship. The ascending line represents increase in response with increasing nutrient intake, while the horizontal line represents nutrient sufficiency. In other words, the second line (i.e. the horizontal) means a plateau in which no statistical significance should exist between the groups along the line. In the present study, weight gain of grouper fed 16\% lipid diet decreased significantly, thus the relationship between fish growth and dietary lipid level is best expressed by a second-order regression curve (Fig. 1) and the maxima of the curve was obtained at about 9\% lipid. Note that weight gain of the fish in the 4\% lipid group was not significantly different from those in the 8\% and 12\% lipid groups (Table 2) suggesting that 4\% dietary lipid may meet the minimum lipid requirement for grouper.

White blood cell (WBC) plays an important role in the immune response in fish, particularly in inflammation (Secombes, 1996). Phagocytes possess a unique membrane enzyme, NAPDH oxidase, capable of reduction of molecular oxygen into superoxide
anion (O₂⁻) during a process termed the respiratory burst. Superoxide anion production is considered to be one of the most important microbicidal components in the armoury of phagocytes (Secombes, 1990). Lysozyme and alternative complement (ACH50) activity are commonly used as humoral immune indicators in fish (Obach et al., 1993; Tort et al., 1996; Montero et al., 1998; Ortuno et al., 2000; Clerton et al., 2001). All these four immune response parameters used in the present study were higher in grouper fed diets containing lipid compared with the fish fed with the lipid-deprived diet, clearly demonstrating that dietary lipid is an immunostimulant for grouper.

An interesting finding of the present study was that high supplementation of lipid (e.g. 16%) depressed growth but not the immune response in grouper. It has been reported that the respiratory burst activity of phagocytes of European sea bass decreased with increasing dietary lipid level from 9% to 17% (Sitjá-Bobadilla and Pérez-Sánchez, 1999). It should be noted, however, that in that study, the protein content decreased as the content of lipid in the diet increased. Thus, the decrease in the immune response (respiratory burst activity) could not be attributed solely to the increased dietary lipid level.

Dietary lipid source has been shown to affect the immune response in channel fish (Fracalossi and Lovell, 1994), rainbow trout (Kiron et al., 1995) and Atlantic salmon (Thompson et al., 1996). The influence of lipid source was mainly due to the amount of essential fatty acids. Wu et al. (2002) recently demonstrated that docosahexaenoic acid (DHA) was superior to eicosapentaenoic acid (EPA) as the essential fatty acid for growth of grouper. In the present study, a 1:1 mixture of fish oil and corn oil was used to provide both n-3 and n-6 fatty acids to prevent the essential fatty acid deficiency. Future studies regarding the essential fatty acid requirements and their effect on immune response in grouper is needed.

Acknowledgements

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References


