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Effects of Dietary Protein and Lipids on Blood Parameters and Superoxide Anion Production in the Grouper, *Epinephelus coioides* (Serranidae: Epinephelinae)

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Ann-Chang Cheng, Chia-Yung Chen, Chyng-Hwa Liou, and Ching-Fong Chang (2006) Effects of dietary protein and lipids on blood parameters and superoxide anion production in the grouper, *Epinephelus coioides* (Serranidae: Epinephelinae). *Zoological Studies* **45**(4): 492-502. The objectives of this study were to investigate the effects of dietary lipid and protein levels on blood parameters, body composition, body indices, and the production of superoxide anions in the grouper, *Epinephelus coioides* (Serranidae: Epinephelinae). The effects of acute cold stress on the production of superoxide anions were also studied. A feeding trial was conducted for 12 wk on 200 juvenile groupers (10 g) per cage placed in a 2-ton tank with a recirculation system. In total, 4 dietary treatments with a 2 x 2 factorial array were conducted. Treatments had either low (L, 31%) or moderate (M, 47%) crude protein (P) combined with either moderate (M, 10%) or high (H, 18%) fat (F). Fish were hand-fed to apparent satiation. Consumption of the high-fat, low-protein (LP-HF) diet resulted in abnormally increased levels of plasma glucose, triglycerides, and cholesterol, lower production of superoxide anions, and poor growth performance, which is consistent with higher body lipid content, viscerosomatic index, intraperitoneal fat, and hepatosomatic index of the fish. However, consumption of the moderate-protein, high-fat (MP-HF) diet decreased the impaired metabolism and increased the production of superoxide anions in the grouper. Grouper fed the high-fat (LP-HF and MP-HF) diets produced significantly higher levels of superoxide anions after acute cold shock (stress) compared to the control fish. In summary, our data suggest that high dietary fat results in increased fat deposition, and plasma triglyceride and glucose levels in grouper. High dietary lipid enhanced the immune response of grouper after acute cold shock.
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Key words: Protein, Fat, Growth, Superoxide anion, Grouper.

Appropriate feed composition is important for growth, disease resistance, and immune activity in aquacultured fish. A nutritionally adequate diet satisfies 3 needs: fuel (chemical energy), the organic raw materials for biosynthesis, and a supply of essential nutrients. Proteins, lipids, and carbohydrates are the critical components of the diet for supplying the carbon skeleton and energy. Fish may convert protein into an energy source if non-protein energy sources (carbohydrates and fats) are not present in sufficient quantities in the diet. Therefore, a certain amount of lipids is usually included in diets as an energy source and also to increase the protein efficiency (Cho and Kaushik 1990, Williams et al. 2003).

It was also reported that increasing the level of dietary lipids beyond about 9%-10% did not improve fish growth rates, but instead reduced the fish's appetite in humpback grouper (*Cromileptes altivelis*) (Williams et al. 2004). High protein requirements (Chen and Tsai 1994, Shiau and Lan 1996) and low utilization of carbohydrates and lipids in groupers have been reported (Shiau and Lin 2001 2002, Lin and Shiau 2003). So it can be inferred that groupers, marine carnivores, are adapted to using protein as the preferred energy

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source over carbohydrates and lipids, thus requiring high levels of dietary protein. Worldwide, there is enormous interest in developing high-performance fish diets, both to improve the efficiency of growth and to reduce N-excretion to the environment (Eng et al. 1989, Naylor et al. 2000). Much progress has been achieved with salmonids in the past 10 yrs by including lipids at 300 g/kg or more in the diet, which has in turn enabled reductions in dietary protein concentrations of from 400 to 500 g/kg body weight (BW). Fish productivity has also improved, and N discharges to the environment have been reduced (Helland and Grisdale-Helland 1998, Rasmussen et al. 2000, Torstensen et al. 2001). Theoretical principles or practical methods of salmonid feed production are employed to develop feed for new marine cultured species in the fish feed industry. However, the inclusion of lipids to replace proteins as an energy source for other fish does not always provide similar results as in salmonids. In our preliminary study, poor growth performance and high plasma triglyceride and glucose levels were observed in fish fed a high-fat, low-protein diet; furthermore, increasing the protein level in the diet seemed to improve this abnormal phenomenon. We thus attempted to investigate whether grouper can utilize lipids as well as salmonids and also to study whether a high-fat (18%), low-protein diet (31% crude protein is low compared to the optimal level of 47%-52% for grouper) will induce any abnormal metabolism and further impair the immune function of grouper.

Feeding inadequately formulated feed to an animal will cause metabolic stress and decrease its resistance to diseases. Immune functions decreased in rainbow trout fed a protein-deficient diet (Kiron et al. 1995). Macrophages (an important index of immune function) are considered to be the main phagocytic cells in fish, and they are also the dominant phagocytes in the head kidneys (Lundén et al. 2002). In fish, like in mammals, stimulation of the phagocyte cell membrane, with accompanying activation of the membrane-associated NADPH-oxidase, initiates increased oxygen consumption and the production of reactive oxygen intermediates (ROIs) with microbicidal activity in a process termed the respiratory burst (Płytycz et al. 1989, Secombes 1996). Superoxide anion production (an important member of the ROI family) is considered to be one of the most important microbicidal components in the action of phagocytes (Secombes 1990).

The poor utilization of high dietary fat in relation to the protein level in grouper, and interest in determining whether abnormal metabolism in fish fed a high-fat, low-protein diet impairs its immune function were the main reasons we conducted the present study. The objectives were to investigate the effects of either low (L, 31%) or moderate (M, 47%) crude protein (P) with moderate (M, 10%) or high (H, 18%) fat (F) on the blood parameters, body composition, body indices, growth, and superoxide anion production in grouper, *Epinephelus coioides*. Since grouper culture often experiences the problem of cold shock during the winter in Taiwan, the effect of cold shock on the production of superoxide anions was also studied.

MATERIALS AND METHODS

Diet preparation

Protein and lipid levels in the diet were prepared according to the studies of Shiau and Lan (1996). In total, 4 dietary treatments with a 2 x 2 factorial array were conducted. Factorial treatments had either low (L, 31%) or moderate (M, 47%) crude protein (P) with moderate (M, 10%) or high (H, 18%) fat (F) (Table 1). All diets were individually blended in a mixer and then homogenized after mixed oil was added. Distilled water was included to achieve a proper pelleting consistency, and the mixture was further homogenized. The mixed ingredients were made into pellets using an extruder (Ming Seng Machinery, Cooperation, Taiwan) with a 2 mm diameter and a rotation cutter. The pelleted diets were dried at 40°C for 10 h then stored at -20°C until used for the feeding trial.

Experimental procedures

Grouper larvae (*E. coioides*) were purchased from the Tungkang Branch of the Fisheries Research Institute, Tungkang, Taiwan and shipped to National Taiwan Ocean University for the experiment. After 1 mo of culture, juvenile fish of 10 g in body weight (n = 800) were selected and randomly divided into 4 culture nets (nets I, II, III, and IV; 200 fish per culture net with dimensions of 60 x 35 x 40 cm). Two of the culture nets were placed in a 2-ton seawater fiber-reinforced plastic (FRP) tank. A recirculatory system was designed for each FRP tank with a nylon filter, biofilter, and deproteinizing devices to remove organic substances and feces. Seawater conditions in the recirculatory system were as follows: a salinity of 32 ppt, water temperature of 28 ± 0.5°C, pH 8.2, dissolved O_2 of 5.5 ±

0.5 mg/l, and NH_3-N of < 0.3 mg/l.

The LP-MF and LP-HF, and MP-MF and MP-HF diets were assigned to the 2 tanks, respectively. The experiments lasted for 12 wk, and the fish were weighed regularly at 2 wk intervals. The diet was given twice a day (at 08:00 and 17:00). At each meal, fish were hand-fed to apparent satiation. After feeding each meal, the uneaten feed was manually removed from the bottom of the FRP tank and dried in an oven to estimate the amount of the uneaten feed in order to calculate the feed consumption. At the end of the experiment (17 h after the last feeding) (Shimeno et al. 1990), 10 fish were randomly selected from each net cage, and immediately placed into a bucket containing 10 L seawater (200 ppm tricaine methanesulfonate, MS-222, Sigma, St. Louis, MO, USA). Blood samples (10 fish per group) were drawn from a caudal vessel immediately after the fish were anesthetized. The sampled fish were sacrificed by an overdose of MS-222. The fish were dissected in order to weigh the viscera, liver, and intraperitoneal fat to determine the hepatosomatic index (HSI), condition factor (CF), viscerosomatic index (VSI), and intraperitoneal fat (IPF). The viscera and body were stored at -20°C for individual body composition analysis (n = 10 per group) (AOAC, 1984). Blood samples were immediately centrifuged, and the plasma was removed and stored at 4°C in a refrigerator until analysis. Different parameters in the plasma were analyzed within 12 h after collection. Weight gain, feed efficiency, and total feed consumption were also calculated. At the end of the experiment, 18 fish from each group were analyzed for superoxide anion production.

For the cold shock experiment, fish from the 4 dietary experimental groups were selected and divided into 2 groups: control (n = 8) and cold stress (n = 8). There were 2 set of separate recirculation systems connected to several aquariums (60 x 30 x 40 cm). Cold shock was separately administered to fish fed the LP-MF and LP-HF diets and to fish fed the MP-MF and MP-HF diets. Sixteen tested fish were moved into an aquarium and fed the test diet for 1 wk to allow acclimation to the new experimental conditions before the experiment. For the cold stress treatment, water

Table 1. Diet formulations and proximate composition in the experiment (% dry matter)

Diet	LP-MF[1]	LP-HF[2]	MP-MF[3]	MP-HF[4]
Ingredient (*g kg*$^{-1}$)				
Brown fish meal	44.5	44.5	60.57	60.57
Starch5	40.47	21.5	19.19	12.06
Wheat gluten	-	-	4.11	4.11
Corn gluten	-	-	1.89	1.89
Oil mixture[6]	6.33	14.30	4.93	12.93
α-Cellulose	-	11.00	0.61	0.16
CMC[7]	2.00	2.00	2.00	1.58
Basal mixture[8]	2.20	2.20	2.20	2.20
Mineral mixture[9]	3.00	3.00	3.00	3.00
Vitamin mixture[9]	1.5	1.5	1.5	1.5
Proximate composition (%)				
Moisture	5.53	4.29	4.25	4.40
Crude protein	31.49	31.55	46.87	46.68
Lipid	9.23	17.72	9.97	17.83
Ash	13.65	13.45	17.30	17.25
ME (kcal/100g diet)[10]	366	367	366	410

[1]LP-MF: low protein and moderate fat. [2]LP-HF: low protein and high fat. [3]MP-MF: moderate protein and moderate fat. [4]MP-HF: moderate protein and high fat. [5]Different starch levels are to adjust equal metabolizable energy in various diets. [6]Lipid source: soy bean oil /cod liver oil (3/7). [7]CMC: carboxymethyl cellulose. [8]Basal mixture: Squid meal 1%; CaHPO4 0.50%; Choline 0.70%. [9]Mineral and vitamin mixture: According to Teng et al. (1978). [10]Metabolizable energy (ME): protein, 4.5 kcal g^{-1}, carbohydrate, 3.49 kcal g^{-1}, lipid, 8.5 kcal g^{-1} (Shiau and Huang 1989).

temperatures were decreased from 28 to 15°C within 4 h with a refrigerator in the recirculatory system, and then the fish were maintained at 15°C for 2 h before the water temperature was returned to 28°C within the next 4 h. After the cold-shock treatment, the stressed fish were maintained at 28°C in the same aquarium for 1 d, and then 8 fish were selected randomly to collect the head kidneys for the assay of superoxide anion production. In the control group, the water temperature was maintained at a constant 28°C throughout the cold-shock experiment.

Blood analysis

Glucose and triglyceride in the plasma were respectively analyzed using glucose and triglyceride kits, according to the manufacturer's instructions (Audit Diagnostics, Cork, Ireland). Similarly, cholesterol and total protein were measured using the respective kits, Cholesterol FL (CT F400 CH) and Proteine Totali (TP 0500 CH) (Chema Diagnostica, Jesi, Italy).

Measurement of superoxide anions

The collected head kidney samples were dispersed in AL medium (Aim V and Leibovitz's L15, GIBCO, Rockville, MD, USA) by repeatedly passing it through a plastic transfer pipette. The mixture was filtered through 100-μm nylon. Percoll (2 ml of 30%-51%; Sigma) was added to the cell suspension and centrifuged at 400 x*g* for 40 min (4°C). Leukocyte layers obtained from the interface were collected and centrifuged again at 300 x*g* for 10 min at 4°C. The leukocyte pellets thus obtained were resuspended in AL-serum medium (AL medium with 10% fetal bovine serum) at a concentration of 1 x 10^7 cell/ml. Leukocytes (1.0 x 10^6 cells in 100 μl) were added to each well of a microfluore plate (Nunc-Immuno Modules, Roskilde, Denmark) and cultured overnight at 25°C in an incubator. Superoxide anion production was assayed by a nitroblue tetrazolium (NBT) reduction assay (Anderson and Brubacher 1995). The NBT solution (100 μl per well, 1 mg of NBT and 1 μg of phorbol 12-myristate 13-acetate in 1 ml of L-15 medium, Sigma) was added to each well and incubated at 30°C for 1 h. The cells were fixed with 100% alcohol, and then 120 μl of KOH (2 M) and 140 μl of dimethyl sulfoxide were added for formazan formation. Superoxide anion production was quantified by the measurement of OD 620 nm.

Statistical analysis

The data are expressed as the mean. Two-way analysis of variance (ANOVA) was used to test the significance of different diets for each parameter, followed by Duncan's multiple range test to determine individual mean differences when a significant effect was found.

RESULTS

Growth index

Table 2 summarizes the values of weight gain, feed efficiency, total feed consumption, and survival of groupers fed the experimental diets for 12 wk. The weight gain and total feed consumption in fish fed the MF diets were higher compared to those fed the HF diets at the same protein level. The MP diets resulted in a higher weight gain and feed efficiency compared to the LP diets at the same fat level. The total feed consumed by fish fed the diets with the same fat level had similar values. The lowest growth performance was obtained in fish fed the LP-HF diet.

Body condition indices

Results of the body condition indices are shown in table 3. HSI, VSI, and IPF were significantly affected by dietary protein and fat, and by the interaction between protein and fat. The 3 indices were significantly higher in fish fed the LP-HF diet than in fish fed the other 3 diets, but there

Table 2. Weight gain, total food consumed and survival of grouper fed different protein and fat levels (LP, low protein; MP, moderate protein; MF, moderate fat; HF, high fat) in the diets for 12 wk

	WG (%)[1]	FE[2]	TFC (%)[3]	Survival (%)
LP-MF	367.47	0.85	344	100
LP-HF	268.54	0.89	241	100
MP-MF	495.92	1.12	346	100
MP-HF	339.64	1.10	243	100

[1]Weight gain (WG) =100 x (Final body weight-Initial body weight)/ Initial body weight. [2]Feed efficiency (FE) = g weight gain/g feed consumed. [3]Total feed consumption (TFC) = total feed consumption /Initial weight.

were no differences between the MP-MF and MP-HF groups; moreover, the VSI and IPF were significantly lower in fish fed the LP-MF diet compared to those of fish fed the other 3 diets. No significant effects of dietary protein, fat, or the interaction between protein and fat on CF were found across the treatments.

Table 3. Hepatosomatic index (HSI), condition factor (CF), viscerosomatic index (VSI) and intraperitoneal fat (IPF) of groupers fed with different protein and fat levels (LP, low protein; MP, moderate protein; MF, moderate fat; HF, high fat) in the diets for 12 wk

Diet	HSI[1]	CF[2]	VSI[3]	IPF[4]
LP-MF	3.29[a]	16.00[a]	9.61[a]	1.92[a]
LP-HF	4.82[c]	16.36[ab]	14.77[c]	3.74[c]
MP-MF	3.63[ab]	16.71[c]	9.88[b]	2.10[b]
MP-HF	3.75[b]	16.18[ab]	9.94[b]	2.22[b]
Two-way ANOVA				
Dietary protein (P)	$p<0.014$	$p<0.917$	$p<0.001$	$p<0.001$
Dietary Fat (F)	$p<0.001$	$p<0.261$	$p<0.001$	$p<0.001$
P x F (Interaction)	$p<0.001$	$p<0.020$	$p<0.001$	$p<0.001$
Pooled S.E.	0.14	0.19	0.08	0.06

Values represent means of ten fish from net cage. Values in a column that do not have the same superscript are significantly different at $p < 0.05$ based on Duncan's multiple range test. [1]HSI = (liver weight / body weight) x 100. 2CF = body weight/(total length)^3 x 1000. [3]VSI = (viscera weight / body weight) x 100. [4]IPF = (intraperitoneal fat weight / body weight) x 100.

Table 4. Body composition (%) of grouper fed with different protein and fat levels (LP, low protein; MP, moderate protein; MF, moderate fat; HF, high fat) in the diets for 12 wk (each value was collected from 10 fish)

Diet	Moisture	Protein	Lipid	Ash
LP-MF	71.38[b]	17.08[bc]	5.33[a]	4.85[c]
LP-HF	70.30[a]	16.62[a]	7.07[d]	4.37[a]
MP-MF	71.52[b]	17.30[c]	5.62[b]	4.68[b]
MP-HF	71.14[b]	16.93[b]	6.16[c]	4.67[b]
Two-way ANOVA				
Dietary protein (P)	$p<0.021$	$p<0.020$	$p<0.001$	$p<0.006$
Dietary Fat (F)	$p<0.001$	$p<0.005$	$p<0.001$	$p<0.001$
P x F (Interaction)	$p<0.079$	$p<0.480$	$p<0.001$	$p<0.001$
Pooled S.E.	0.14	0.09	0.03	0.02

Values represent means of ten fish from net cage and are expressed on a wet-weight basis. Values in a column that do not have the same superscript are significantly different at $p < 0.05$ based on Duncan's multiple range test.

Body composition

The compositions of the whole body are shown in table 4. The moisture, protein, lipid, and ash contents of the whole body were significantly affected by dietary protein and fat, while a significant interaction between protein and fat was only found for lipid and ash contents of the whole body. The lipid content was significantly higher, while the moisture, protein, and ash contents of the whole body were significantly lower in fish fed the LP-HF diet compared to the other 3 diets. The lipid content in fish fed the LP-MF diet was the lowest across the 4 treatments.

Blood parameters

Results in table 5 indicate that 17 h after the last feeding, the concentrations of plasma glucose and triglyceride were significantly affected by dietary protein and fat, but the concentration of plasma cholesterol was only significantly affected by dietary fat. No dietary protein or fat effect on total plasma protein was observed. The significant interaction between dietary protein and fat was only observed in the results for plasma triglyceride. There was a significant increase in plasma triglyceride, glucose, and cholesterol levels in fish fed the diets containing higher dietary fat level (HF vs. MF) at the same dietary protein level. The plasma triglyceride level increased by 5-fold in the LP-HF group compared to the LP-MF group and by 3-fold in the MP-HF group compared to the MP-MF group. Plasma glucose (1.7 fold) and cholesterol (3-fold) concentrations significantly increased with the higher dietary fat level but the same protein level. However, there was a significant decrease in plasma triglyceride and glucose levels in fish fed the diets containing the higher dietary protein level (MP vs. LP) at the same dietary fat level. The plasma triglyceride level decreased to 64% in the MP-MF group compared to the LP-MF group and to 40% in the MP-HF group compared to the LP-HF group. Plasma glucose (70%) concentrations significantly decreased with an increasing dietary protein level at the same dietary fat level.

Effects of diets on the production of superoxide anions

The production of superoxide anions was sig-

nificantly affected by dietary protein and fat, and the interaction between protein and fat (Table 6). The highest production of superoxide anions was observed in fish fed the MP-HF diet while the lowest production was observed with the LP-HF diet. There was no significant difference in the production of superoxide anions between fish fed the LP-MF and MP-HF diets (Table 6).

Among the fish fed the different dietary fat levels at the same protein level, the production of superoxide anions in fish fed the LP-HF diet was significantly lower than that of fish fed the LP-MF diet, but fish fed the MP-HF diet had significantly higher production of superoxide anions than fish fed the MP-MF diet (Table 6). Similarly, in fish fed different protein levels at the same fat level, the production of superoxide anions of fish fed the MP-MF diet was significantly lower than that of fish fed the LP-MF diet, but fish fed the MP-HF diet had significantly higher production of superoxide anions than fish fed the LP-HF diet (Table 6).

Effects of acute cold stress on the production of superoxide anions

Figure 1 shows the effect of acute cold shock on superoxide anion production in fish fed the LP with MF and HF (Fig. 1A) and the MP with MF and HF (Fig. 1B) diets, respectively. Superoxide anion production was found to be higher in the cold-shocked group compared to the control group in the LP-HF group, but there was no significant difference in fish fed the LP-MF diet (Fig. 1A). Superoxide anion production was higher in the cold-shocked group compared to the control group in fish fed the MP-HF diet, but it was significantly lower in the cold-shocked group than in the control group in fish fed the MP-MF diet (Fig. 1B).

DISCUSSION

In the present study, 200 groupers were placed in a cage and fed to satiation twice per day. After 12 wk, the FE ranged from 0.85 to 1.10 and survival was 100% for all treatments, which indicated that the feeding trial was conducted well. Due to the limitation of replication in the statistics, growth results obtained in the present study are only suggested to be evidence to support the results of data from healthy grouper cultured in a high-quality environment. However, growth performance in the present study is consistent with our previous results (unpubl. data). In the present study, our results showed that a diet with a moderate dietary protein content (MP-MF and MP-HF) resulted in better weight gain and feed efficiency compared to the low-protein diets; high dietary

Table 5. Plasma glucose, plasma triglyceride, plasma cholesterol and plasma total protein concentrations (mg/100 ml) of groupers fed with different protein and fat levels (LP, low protein; MP, moderate protein; MF, moderate fat; HF, high fat) in the diets for 12 wk

Diet	Glucose	Triglyceride	Cholesterol	Total protein
LP-MF	120.5^{b}	493.9^{b}	263.3^{a}	5.0
LP-HF	208.1^{d}	2409.4^{d}	748.1^{b}	4.7
MP-MF	84.1^{a}	315.1^{a}	250.0^{a}	4.9
MP-HF	145.3^{c}	954.4^{c}	758.0^{b}	4.9
Two-way ANOVA				
Dietary protein (P)	$p<0.001$	$p<0.001$	$p<0.770$	$p<0.785$
Dietary fat (F)	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.625$
P x F (Interaction)	$p<0.073$	$p<0.001$	$p<0.710$	$p<0.815$
Pooled S.E.	6.97	51.34	19.66	0.14

Values represent means of ten fish from net cage. Values in a column that do not have the same superscript are significantly different at $p < 0.05$ based on Duncan's multiple range test.

Table 6. Superoxide anion production (respiratory burst activity, expressed as OD620nm) of leucocytes isolated from the head kidney of juvenile grouper fed with different levels of protein and fat (LP, low protein; MP, moderate protein; MF, moderate fat; HF, high fat) for 12 wk

Diet	SAP
LP-MF	0.6948^{a}
LP-HF	0.4576^{c}
MP-MF	0.6287^{b}
MP-HF	0.7212^{a}
Two-way ANOVA	
Dietary protein (P)	$p<0.001$
Dietary fat (F)	$p<0.001$
P x F (Interaction)	$p<0.001$
Pooled S.E.	0.02

Superoxide anion production (mean ± standard deviation, SD) from the leukocytes in the head kidney of groupers (n = 18 in each group) fed with different diets, LP-MF and LP-HF, MP-MF and MP-HF for 12 wk. Values in a column that do not have the same superscript are significantly different at $p < 0.05$ based on Duncan's multiple range test.

lipids (LP-HF and MP-HF) resulted in decreased feed consumption and weight gain. This is consistent with the study of Lin and Shiau (2003) who reported decreased growth and feed efficiency of Malabar grouper (*Epinephelus malabaricus*) fed a high-lipid diet. It was also reported that increasing the dietary lipid beyond about 9%-10% did not improve fish growth rates but instead reduced the fish appetite in humpback grouper (*Cromileptes altivelis*) (Williams et al. 2004). Similar findings were also observed in other fish species (Sanz et al. 1993, Kaushik and Médale 1994). However, dietary lipids (10%-20%) have been reported to facilitate the protein efficiency and growth performance in arctic charr (*Salvelinus alpiinus* L.) (Tabachek 1986), yellowtail (*Seriola quinqueradiata*) (Takeuchi et al. 1992), and rainbow trout (*Salmo gairdneri*) (Beamish and Medland 1986). However, the inclusion of high lipids in the diet did not increase the feed efficiency in grouper; thus a low utilization of lipids in grouper is suggested.

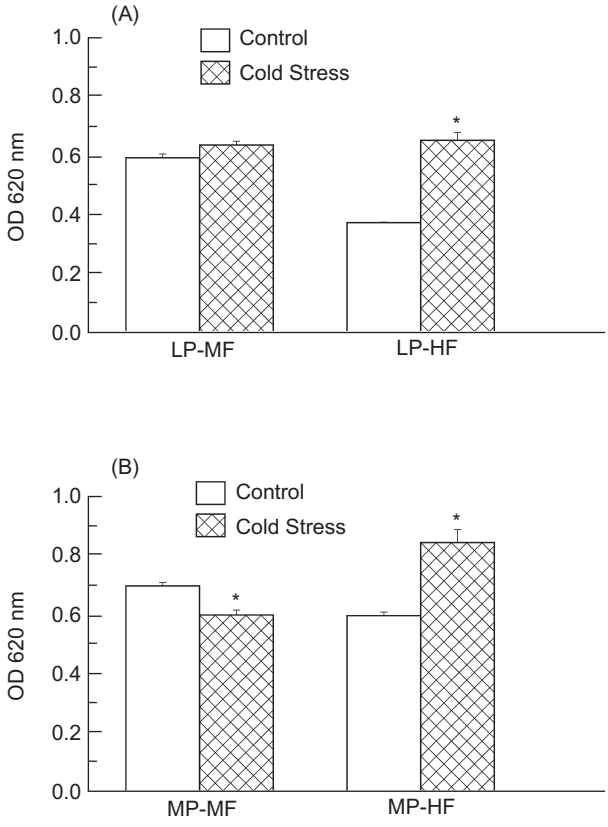

Fig. 1. Superoxide anion production (optical density, OD 620 nm) by leukocytes in the head kidneys of grouper (n = 8 in each group) fed different protein and fat levels in the diets (LP-MF and LP-HF, and MP-MF and MP-HF) for 12 wk. The fish were divided into control (non-stressed status) and cold-stress groups (fish were stressed at 15°C for 2 h and the head kidneys were collected 17 h later). LP-MF (low protein and moderate fat), LP-HF (low protein and high fat), MP-ML (moderate protein and moderate fat), and MP-HF (moderate protein and high fat). *Significantly different compared to the control (p < 0.05).

In this study, the significantly decreased lipid and increased protein and moisture contents of the whole body were observed in grouper fed the diets containing higher dietary protein (MP vs. LP) at the same dietary fat level. Similar results were observed in olive flounder (*Paralichthys olivaceus*) (Kim et al. 2004), *Spinibarbus hollandi* (Yang et al. 2003), and silver perch (*Bidyanus bidyanus*) (Yang et al. 2002). Higher dietary fat resulted in an increase in the body lipid content. Similar findings have been reported in other species such as tilapia (*Tilapia zilli*) (El-Sayed 1987), European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles 1999), and rainbow trout (*Oncorhynchus mykiss*) (Jobling et al. 1998). However, in our previous study (unpubl. data), the body lipid content of grouper increased with an increasing level of lipids in the diet, but the effect was not obvious in the muscle. This is because the lipid content in muscle ranged from 1.5% to 2.5% (in wet weight) despite the high lipid level in the diet. In the present study, the higher lipid content of the whole body of fish fed the LP-HF diet might be attributed to the high lipid accumulation in the cavity of the abdomen, which is consistent with the high level of the viscerosomatic index, intraperitoneal fat, and hepatosomatic index of the fish. The higher-lipid diet also produced a high HSI possibly because of the high lipid content in the liver which is similar to observations in gilthead seabream (*Sparus aurata* L.) (Santinha et al. 1999), haddock (*Melanogrammus aeglefinus*) (Nanton et al. 2000), and Atlantic cod (*Gadus morhua* L.) (Morais et al. 2001). There was a significant interaction of lipid content of the whole body with dietary protein and fat in the fish, which was not found in juvenile masu salmon (*Oncorhynchus masou* Brevoort) (Lee and Kim 2001) or Atlantic salmon (*Salmo salar*) (Refstie et al. 2001).

Significant increases in plasma triglyceride, cholesterol, and glucose levels were found in grouper fed the high-fat (18%) diets in the present study. Furthermore, high plasma triglyceride and cholesterol levels (3-5 fold increases) were found in grouper fed the high-fat diets as compared to the moderate (10%)-fat diets. This shows that grouper is inefficient at utilizing fat in its diet. A similar observation was also found in turbot (*Psetta maxima*) (Regost et al. 2001), a lean fish (with a low lipid content in the muscle). But high

plasma triglycerides were not found in salmon (*Salmo salar*) fed diets containing 31%-47% fat (Hemre and Sandnes 1999). Higher plasma glucose levels (up to a 1.7-fold increase) were detected in grouper fed the high-fat diets as compared to the moderate fat groups (LP-HF vs. LP-MF and MP-HF vs. MP-MF) 17 h after the final feeding. According to our preliminary time-course experiment and the results of Shimeno et al. (1990), it is considered appropriate to collect blood 17 h after the final feeding for the plasma glucose assay. Higher dietary starch resulted in higher plasma glucose levels in smaller salmon (*Salmo salar* L.) (Hemre et al. 1996). However, in this study the included starch level (40.5%) in the LP-MF diet was about 2 fold the level (21.5%) of that in the LP-HF diet, but the plasma glucose of fish fed the LP-HF diet was 1.7 fold that of fish fed the LP-MF diet. The plasma glucose level (84 mg/dl) in fish fed the MP-MF diet was close to the level (72 mg/dl) in the study reported by Luo et al. (2005), in which they conducted a study for 8 wk with the same species of grouper, fed the fish twice to satiation with a diet similar to the MP-MF diet, and collected blood samples 18 h after the final feeding. The higher plasma glucose levels in this study were attributed the poor utilization of carbohydrates by the grouper compared to tilapia (*Oreochromis niloticus* x *O. aureus*) (Lin et al. 2000) and the regime of feeding to satiation rather than fixed rations (unpubl. data). Therefore, it is reasonable to speculate that the higher plasma glucose was due to the effect of high dietary fat. Studies have suggested that the reason for type 2 diabetes is partly due to the high dietary lipids in mice (Zhang et al. 2001). Similar to the symptoms of type 2 diabetes (Boden and Shulman 2002), the blood triglyceride, glucose, and cholesterol levels in fish fed the LP-HF diet were obviously high in this study. In contrast to the result that plasma triglyceride and glucose increased in grouper fed the higher-fat diets (HF vs. MF: 18% vs. 10%), significant decreases in plasma triglycerides and glucose were observed in grouper fed the higher-protein diets (MP vs. LP: 47% vs. 31%). The effect of higher dietary protein of decreasing plasma glucose was observed in Arctic charr (*Salvelinus alpinus*) (Cameron et al. 2002) and on decreasing plasma triglycerides was found in African catfish (*Clarias gariepinus*) (Matter et al. 2004). Thus, the highest plasma triglyceride and glucose in fish fed the LP-HF diet can possibly be explained by it containing the lowest dietary protein and the highest dietary fat among the 4 treatments. The significant interactive effect between protein and fat on plasma triglycerides might also have contributed to the obviously high plasma triglycerides in fish fed the LP-HF diet. Our results suggest that the abnormal metabolism of glucose and triglycerides in grouper might be caused by high dietary fat and low protein in the diet. The results also imply that moderate protein in the diet may decrease the adverse effects of high dietary fat on lipid and glucose metabolism in the grouper.

Superoxide anion production is considered to be one of the most important microbicidal components and is an important index of immune function (Secombes 1990). After 12 wk of the feeding trial, the grouper fed the LP-HF diet had the lowest production of superoxide anions, along with poor growth performance and significantly higher blood parameters (glucose and triglycerides), body lipid content, and body condition indices across the 4 treatments. There are contradictory reports about the effects of high dietary fat on immune function: a high fat diet increased the phagocytic ability in channel catfish (*Ictalurus punctatus*) (Sheldon and Blazer 1991); fat (50% fish oil and 50% corn oil) supplementation of up to 16% in a 50% protein diet did not inhibit superoxide anion production in grouper (*E. coioides*) (Lin and Shiau 2003); decreased respiratory bursts were found in sea bass (*Dicentrarchus labrax* L) fed a high-fat diet (17%) (Sitjà-Bobadilla and Pérez-Sánchex 1999); and an increase in highly unsaturated fatty acids in the diet resulted in increased phagocytosis and respiratory bursts (superoxide anion production) in the grouper (*E. coioides*) (Wu et al. 2002). The lower dietary protein and the interaction between protein and fat in this study compared to other studies might be the reasons for the difference in superoxide anion production of fish fed the high dietary fat. In this study, decreased superoxide anion production (respiratory bursts) was found in grouper fed the LP-HF diet compared to those fed the LP-MF diet. In contrast, an increase in superoxide anion production (respiratory bursts) was observed in grouper fed the MP-HF diet compared to those fed the MP-MF diet The contradictory result might be explained by the significant interaction between dietary protein and fat. These results suggest that high-fat and low-protein contents together in a diet are factors which can reduce the production of superoxide anions in grouper.

Low water temperatures may disturb physiological homeostasis, especially in a warm-water fish species such as the grouper (Le Morvan et al. 1998). The effect of temperature on the immune

system in fish has been extensively discussed (Bly and Clem 1992, Zapata et al. 1992), with the production of superoxide anions also being mentioned. The cold-shock treatment in this study indicated that high dietary fat increased superoxide anion production as compared to the control; however, it was not increased in fish fed the moderate-fat diets. Highly unsaturated fatty acids in the diet are important in fish to increase membrane permeability and fluidity, thus enabling the fish to adapt to low water temperatures (Malak et al. 1989, Xu et al. 1996). Therefore, the increase in the production of superoxide anions in fish fed high dietary fat after cold shock can be also attributed to the increased polyunsaturated fatty acids (PUFAs:eicosapentaenoic acid and docosahexaenoic acid) in the diets (HF = 2.75% vs. MF = 1.6% of PUFA) contents in the diets instead of the increased dietary fat level. Bly et al. (1993) examined immunosuppression when the water temperature was severely reduced during a 24 h period but not after acclimation (Bly and Clem 1991). Płytycz and Jurewicz (1994) observed much-higher efficiency of endocytosis and phagocytosis when fish were first acclimated to cold temperatures as compared to non-acclimated fish. The different immune responses in those studies may have depended on whether the fish were immediately examined for immune function or after acclimating for a while after cold treatment. Higher production of superoxide anions was observed in stressed fish fed the diets (LP-HF and MP-HF) containing high dietary fat (also high PUFA contents) as compared to the control group, respectively. In our study, after cold-shock treatment, the stressed fish were allowed to recover for 1 d at 28°C, and then were dissected to assay superoxide anion production (respiratory bursts). Under this experimental condition, the stressed fish might have become acclimatized to the change in temperature, and the cold shock might have induced an increase of respiratory bursts instead of their suppression.

In summary, our data suggest that high dietary fat results in increases in fat deposition, plasma triglycerides, and glucose in grouper, and it was more pronounced with low protein in the diet, but moderate protein in the diet reduced the adverse effects of high dietary fat in grouper. The high-fat, low-protein diet resulted in an obviously deceased production of superoxide anions. High dietary fat (containing a high PUFA content) enhanced the production of superoxide anions in grouper after cold shock.

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