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Research Article

Effects of dietary protein, lipid and carbohydrate levels on hematological parameters, intestinal histoarchitecture and digestive enzymes activities in orange-spotted grouper (*Epinephelus coioides*) juveniles

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Abstract

A 56-day research was conducted to assess the influence of dietary protein content and carbohydrate: lipid ratio on growth performance, hematological parameters, intestinal histoarchitecture and digestive enzymes activities of orange-spotted grouper (*Epinephelus coioides*) juveniles (13.7 g). Four experimental diets were designed with various protein, lipid and carbohydrate levels namely LP-LCL (low protein 40%−low carbohydrate: lipid ratio=1.4), HP-LCL (high protein 48%−low carbohydrate: lipid ratio=1.4), LP-HCL (low protein 40%−high carbohydrate: lipid ratio=2.9) and HP-HCL (high protein 48%−high carbohydrate: lipid ratio=2.9). Fish were stocked into twelve 300-L cylindrical polyethylene tanks (15 fish in each tank) and fed with experimental diets. The low carbohydrate: lipid ratio diets improved growth rate of the fish. The lipid content of the whole body increased with decreasing dietary carbohydrate to lipid ratio. The intestinal folds' length and thickness significantly increased in the low carbohydrate: lipid ratio treatments. The intestinal total alkaline protease activity elevated by increasing dietary protein content, meanwhile the activities of lipase and amylase increased with increasing dietary lipid and carbohydrate levels, respectively. Blood hematocrit decreased but hemoglobin increased by increasing dietary carbohydrate to lipid ratio. According to the results dietary lipid is more preferable than carbohydrate as an energy source for orange-spotted grouper juveniles and induces protein sparing effect in a low protein content diet.

Keywords: Dietary macronutrients, Digestive enzymes, Enterocytes, Grouper, Growth, Hematology

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Introduction

It has been well known that carnivorous fish species utilize dietary protein more efficient than lipid and carbohydrate for growth and energy production. However, high level of dietary protein enhances feed costs, increase nitrogenous waste and reduce protein efficiency ratio (NRC, 2011). Using non-protein energy sources (NPES) including lipid and carbohydrate for reducing dietary digestible protein to digestible energy ratios leads to protein sparing effect and improve growth, protein productive value, and feed utilization in fish (Ozorio *et al.,* 2006; Darias *et al.,* 2015). Lipids are energy-dense macronutrients among many carnivorous fish species that have limited ability to utilize carbohydrate and it provides lipid-soluble vitamins, phospholipids and essential fatty acids (NRC, 2011). But in some fish species, inappropriate dietary lipid level may reduce feed's pelleting quality, induce excess fat deposition in carcass, which disadvantageous to the shelf-life of the products, suppress feed intake and nutrients efficiency, retard growth rate and provoke oxidative stress in fish (Lin and Shiau, 2003). On the other hand, carbohydrate is an inexpensive energy source, has higher availability and as binder can improve pellet quality as well as it spares protein and L for somatic growth (NRC, 2011). But, extra levels of carbohydrate in diet especially for carnivorous fish species with restricted ability in metabolizing glucose may induce hyperglycemia, poor growth and feed efficiency, extra deposition of glycogen or fat infiltration in the liver, stress intolerance and metabolic burden that eventually compromise immune-competence in fish (Hemre *et al.,* 2002; Vielma *et al.,* 2003; Amoah *et al.,* 2008; Alexander *et al.,* 2011). Furthermore, it has been confirmed that high dietary carbohydrate level result in immunosuppression and oxidative stress in fish (Hemre *et al.,* 2002; Alexander *et al.,* 2011; Zhou *et al.,* 2014; Mozanzadeh *et al.,* 2017; Zhao *et al.,* 2021).

In this sense, it has been reported that dietary carbohydrate to lipid or protein ratios can affect growth performance (Rueda-Jasso *et al.,* 2004; Mozanzadeh *et al.,* 2017), and feed efficiency (Han *et al.,* 2014; Wang *et al.,* 2014) and digestive enzyme activity in various fish species such as Siberian sturgeon (*Acipenser baerii*, Babaei *et al*., 2017), golden pompano (*Trachinotus ovatus*, Dong *et al*., 2018) and hybrid grouper (Gao *et al*., 2018). Also, hematological factors (Wang *et al*., 2014; Mozanzadeh *et al*., 2017) and gut health (Zhao *et al*., 2021) of fish could be significantly affected by dietary carbohydrate to lipid ratios. Thus, providing nutritionally balanced and cost-effective feeds for a fish species, not only supports growth and health status, but also is environmentally friendly by reducing nitrogen loss and water pollution (Kaushik and Medale, 1994; Wu and Gatlin 2014).

The orange-spotted grouper, *Epinephelus coioides,* is considered to be a potential candidate for developing cage culture in many Asian countries because of its high growth rate and high price. A plethora of research have been conducted to determine its nutritional requirements (Reviewed by Williams, 2009). Also, this species is used as a model for monitoring water pollution in its natural habitats (Savari *et al.*, 2020). According to the studies by Luo *et al*. (2004, 2005) the optimum amount of dietary protein, lipid and energy for this species have estimated to be 48%, 10% and 17.5 MJ kg^{-1}, respectively. Marammazi *et al.* (2013) estimated dietary protein and energy content for *E. coioides* fingerlings as 50% and 16 MJ kg^{-1}, respectively. Moreover, Cheng *et al*. (2006) reported that moderate levels of protein (46%) and lipid (10%) significantly improved growth performance and health indices (serum biochemical parameters) in this species. In addition, Wang *et al*. (2017) demonstrated that the optimum dietary carbohydrate: lipid ratio for juvenile Orange-spotted grouper is 0.5 in a diets contained 45% protein and this species is better adapted to utilizing lipids rather than carbohydrates. But, Liu *et al*. (2020) revealed that the optimum dietary carbohydrate: lipid ratio in sub-adult fish is 6.06 in a diet contained 50% protein for grouper culture concerning growth performance and health. In these studies, dietary protein levels were neglected as optimum dietary carbohydrate: lipid ratio was determined, because protein is the source of energy and essential amino acids. Thus, the appropriate levels of dietary protein to energy ratio also should be evaluated in fish. Thus, this research was considered simultaneous effects of dietary protein and carbohydrate: lipid ratios on growth, whole body compositions, gut histoarchitecture, hematological parameters and digestive enzymes activities of the orange-spotted grouper juveniles.

Materials and methods

Experimental setup

The current experiment was done at the Aquatic Research Laboratory of Persian Gulf University (Bushehr, Iran). One hundred and eighty orange-spotted grouper juveniles (13.7±0.6 mean±SE) were transported form marine fish Aquaculture Research Station (Khuzestan, Iran) and acclimatized with the husbandry system for two weeks and fed with a commercial diet (480 g kg^{-1} crude protein, 160 g kg^{-1} crude fat, 100 g kg^{-1} ash and 100 g kg^{-1} moisture, 2 mm size, Beyza Feed Mill 21). Then, fish were stocked into twelve 300-L cylindrical polyethylene tanks (15 fish in each tank). Triplicate groups of orange-spotted grouper were fed with the experimental diets five times a day (0800, 1000, 1200, 1500 and 1800 h) to visual satiation the feeds for 56 days. Tanks were filled with sand-filtered and disinfected (chlorinated (20 ppm) for a day and neutralized with sodium thiosulfate (10 ppm) then UV treated) seawater. About 50% of water of the experimental tanks was exchanged every day. The physicochemical parameters of water including temperature, salinity, pH and dissolved oxygen were 22.0±0.5ºC, 41.0±0.2‰, 8.0±0.1 and 5.6±0.5 ppm, respectively and the photoperiod was 12 h light: 12 h darkness.

Diet preparation

Four experimental diets were formulated (Lindo software, Ver 6.1, USA) with different protein, lipid and carbohydrate levels namely LP-LCL (low protein 40%−low carbohydrate: lipid ratio=1.4), HP-LCL (high protein 48%−low carbohydrate: lipid ratio=1.4), LP-HCL (low protein 40%−high carbohydrate: lipid ratio=2.9) and HP-HCL (low protein 48%−high carbohydrate: lipid ratio=2.9) (Table 1). First, dry feedstuffs were mixed for 15 min and then oils and soybean lecithin were poured on them and blended again for 15 min. Distilled water was added to the mixture for making a dough, then the mixed ingredients were passed through a meat grinder to produce pellets with 2 mm size. Pellets were dried at 60°C for a day, then kept in a freezer (-18ºC).

Table 1: Ingredients and proximate composition of the experimental diets (%).

Ingredients[a]	**LP-LCL**	**HP-LCL**	**LP-HCL**	**HP-HCL**
Fish meal[1]	34.16	40.0	33.53	35.88
Soybean meal[2]	12.0	28.0	20.0	30.0
Wheat gluten[1]	15.01	13.37	5.73	11.92
Wheat meal[1]	19.33	6.78	31.47	16.14
Fish oil+ soybean oil (3:1)[2]	11.25	8.6	6.02	2.81
Soy lecithin[2]	1.0	1.0	1.0	1.0
Vitamin and mineral premixes[3]	2.0	2.0	2.0	2.0
Antioxidant[1]	0.25	0.25	0.25	0.25
Cellulose	5.0	0	0	0
Biochemical composition (%)				
Moisture	6.5	4.2	4.9	4.5
Ash	10.9	9.6	10.1	10.4
Crude protein	40.1	48.3	38.6	46.3
Crude lipid	17.5	15.54	11.83	10.0
Carbohydrate	25.0	22.36	34.57	28.8
CHO:L	1.4	1.4	2.9	2.9
Gross energy (kJ g^{-1})[4]	20.7	21.4	19.6	19.9
P:GE ratio	19.3	22.2	19.4	23.3

Composition of ingredients as % dry-weight basis [Fish meal (65% crude protein, 12% crude lipid); soybean meal (45% crude protein 2% crude lipid); wheat Gluten (72% crude protein, 1.5% crude lipid); wheat meal (45% crude protein, 2% crude lipid).

[1] Beyza Feed Mill, Fars, Iran.

[2] Kesht Va Sanat Shomal Vegetable oil Factories Complex, Neca, Iran.

[3] Vitamin and Mineral premix U kg−1 of premix: vitamin A, 5,000,000 IU; vitamin D3, 500,000 IU; vitamin E, 3000 mg; vitamin K3, 1500 mg; vitamin B1, 6000 mg; vitamin B2, 24,000 mg; vitamin B5, 52,000 mg; vitamin B6, 18,000 mg; vitamin B12, 60,000 mg; Folic acid, 3000 mg; nicotinamide 180,000 mg; antioxidant, 500 mg, copper, 3000 mg; zinc, 15,000 mg; manganese, 20,000 mg; Iron, 10,000 mg; potassium iodate, 300 mg, career up to1 kg, Beyza Feed Mill, Fars, Iran.

[4] Calculated on gross energy values of 23.6 kJ g-1 proteins, 39.5 kJ g-1 lipid and 17.2 kJ g-1 carbohydrates (NRC 1993).

Sample collection

At the end of 56-day husbandry trial fish were fasted. Fish from each tank was individually weighted (g) and their length was determined (mm). Three fish from each tank was anesthetize with 2-

phenoxyethanol (150 ppm) then their blood was collected with heparinized syringe from the caudal vein for hematological evaluation (Mozanzadeh *et al*., 2017). Three fish of each tank were sacrificed and transferred into a -20ºC freezer for determination of the whole body biochemical composition. In addition, three fish of each tank was sacrificed and their foregut was dissected and transferred into formalin buffer (10%, pH: 7.4) for assessing the histoarchitecture of the enterocytes.

Biochemical and histological analyses

The standard methods described by Association of Official Analytical Chemists (2005) were used for evaluating the biochemical composition of the experimental feeds and the whole body of fish. Microscopic studies were done as described by Sotoudeh and Mardani (2018). Histomorphometric parameters including mucosal fold length, mucosal fold thickness, enterocyte height and muscularis thickness at foregut section on ten different villi per fish.

Digestive enzymes and hematological analyses

For evaluating digestive enzyme activity in the gut, after 24 hours fasting, three fish per treatment were sacrificed with the overdose of the same anesthetic and instantly gutted on a piece of ice and the gut and the liver dissected and transferred into separate tubes and kept at −80°C. The gut samples were homogenized (1:30, W: V) in an ice-cold buffer (50 mM mannitol, 2 mM Tris–HCl buffer, pH 7.0) (Gisbert *et al.*, 2016), then they were centrifuged for three min (3300 g, 4°C) and the supernatant was separated for evaluating pancreatic digestive enzymes. The assessment of total alkaline proteases (Walter, 1984) α-amylase (Métais and Bieth 1968), bile salt-activated lipase (Iijima *et al.*, 1998) and the soluble protein (Bradford, 1976) were done according to standard protocols.

The blood samples were suspended in heparinized tube, and white blood cell (WBC) and red blood cell (RBC) counts were then determined after dilution with Natt-Herrick's staining solution. Haemoglobin concentration (Hb) and haematocrit (Ht) were estimated by photometric assay of cyanomethemoglobin and microhematocrit method, respectively as described by Blaxhall and Daisley (1973) and blood indices were calculated according to the following formulae (Dacie and Lewis, 2001):

MCV: mean cell volume = hematocrit (%) / red blood cell count ($\times 10^6$ μL^{-1}) $\times 10$

MCH: mean cell hemoglobin = hemoglobin (g dL^{-1}) / red blood cell count ($\times 10^6$ μL^{-1}) $\times 10$

MCHC: mean cell hemoglobin concentration = hemoglobin (g dL^{-1}) / hematocrit (%)

Statistics

Data were analyzed using the SPSS ver. 16.0 (Chicago, IL, USA). After confirmation of normality and homogeneity of data by Kolmogorov-Smirnov and Leven tests, respectively the effects of dietary protein and carbohydrate: lipid and their interaction effect were determined using a two-way ANOVA. Any significant differences between groups was evaluated with Tukey test (p<0.05).

Results

There was not any mortality during the fish husbandry trial (Table 2). Growth performance parameters including weight gain (p<0.004) and specific growth rate (p<0.004) were significantly affected by dietary carbohydrate: lipid ratio and fish fed low carbohydrate: lipid (1.4) ratio diets had higher growth rate than those fed on high carbohydrate: lipid (2.9) ratio diets. Feed intake (FI) enhanced in fish fed on the high protein content diets (p<0.025).

Table 2: Growth performance and feed utilization in *E. coioides* juveniles fed experimental diets for eight weeks (mean±SE). A different superscript in the same row denotes statistically significant differences (p<0.05).

Parameters	Treatments				Two-Way ANOVA		
	LP-LCL	HP-LCL	LP-HCL	HP-HCL	Protein	CHO/L	Protein × CHO/L
WG (%)[a]	75.6 ± 11.0[a]	80.5 ± 9.9[a]	30.7 ± 19.3[b]	54.7 ± 18.2[ab]	0.137	0.004	0.307
SGR (% initial BW day^{-1})[b]	1.1 ± 0.1[a]	1.2 ± 0.1[a]	0.5 ± 0.3[b]	0.9 ± 0.2[ab]	0.139	0.005	0.272
FI (%)[c]	1.8 ± 0.2[ab]	1.5 ± 0.2[b]	1.9 ± 0.0[a]	1.7 ± 0.1[ab]	0.025	0.068	0.551
FCR[d]	1.6 ± 0.3[b]	1.3 ± 0.3[a]	1.7 ± 0.6[b]	2.1 ± 0.6[c]	0.899	0.042	0.249
PER[e]	1.1 ± 0.3	1.2 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.846	0.079	0.945
PPV[f]	23.9 ± 5.1	23.4 ± 4.8	19.6 ± 3.9	17.7 ± 4.7	0.711	0.167	0.843
K (%)[g]	2.1 ± 0.1	2.2 ± 0.2	1.8 ± 0.5	2.1 ± 0.1	0.348	0.366	0.713
HSI (%)[h]	2.1 ± 0.3[ab]	1.6 ± 0.5[b]	3.1 ± 0.2[a]	3.1 ± 1.1[a]	0.512	0.009	0.634
VSI (%)[i]	10.8 ± 0.6[b]	10.1 ± 0.7[b]	12.0 ± 0.7[a]	10.6 ± 0.4[b]	0.024	0.042	0.33
Survival (%)[j]	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	1.000	1.000	1.000

Diet abbreviations are as follows: LP-LCL, low protein-low carbohydrate to lipid ratio; HP-LCL, high protein- low carbohydrate to lipid ratio; LP-HCL, low protein-high carbohydrate to lipid ratio; HP-HCL, high protein-high carbohydrate to lipid ratio.

[a]WG: weight gain (%) = ((final body weight – final body weight)/ initial body weight) × 100

[b] SGR: specific growth rate (%) = ((ln final body weight – ln final body weight) / t) × 100, where t is experimental period = 56 days.

[c] Feed intake = (dry feed consumed / [(final body weight + initial body weight) / 2] / t) × 100

[d]FCR: feed conversion ratio = feed intake (g) / weight gain (g)

[e]PER: Protein efficiency ratio = protein gain (g) / protein fed (g).

[f]PPV: Protein productive value = retained protein (g) / protein fed (g) × 100

[g]K: Fulton's condition factor = (final body weight / standard length^3) × 100

[h]HSI=(liver weight (g) / whole body weight (g)) × 100

[i]VSI=(visceral weight (g) / whole body weight (g)) × 100

[j]survival (%)=number of fish in each group remaining on day 42/initial number of fish) × 100

The feed conversion ratio (FCR) rose in fish fed on the high carbohydrate: lipid diets (p<0.042). Protein efficiency ratio (PER) and protein productive value (PPV) were not statically different among groups, but their values were improved in fish fed on the low carbohydrate: lipid diets. Hepatosomatic index (HSI) increased in fish fed on the high carbohydrate: lipid diets (p<0.009), meanwhile viscerosomatic index (VSI) was greatest in fish fed on LP-HCL diet and affected by both dietary protein level (p<0.024) and carbohydrate: lipid ratio (p<0.042). Whole body lipid content enhanced with reduction of dietary carbohydrate: lipid ratio (p<0.006), whereas other biochemical parameters were not affected by experimental feeds (Table 3).

Table 3: Whole body proximate composition (%, mean±SE, n=3) of *E. coioides* juveniles fed experimental diets for eight weeks. A different superscript in the same row denotes statistically significant differences (*p*<0.05).

Parameters	Treatments				Two-Way ANOVA		
	LP-LCL	HP-LCL	LP-HCL	HP-HCL	Protein	CHO/L	Protein × CHO/L
Protein	16.9 ± 1.4	17.4 ± 1.0	15.4 ± 0.8	16.1 ± 1.0	0.379	0.054	0.925
Lipid	7.9 ± 0.5[a]	7.7 ± 1.5[a]	6.1 ± 0.2[b]	5.9 ± 0.5[b]	0.720	0.006	0.931
Ash	3.5 ± 0.8	3.2 ± 0.9	3.3 ± 0.7	3.4 ± 0.6	0.829	0.994	0.789
Moisture	70.7 ± 0.5	69.5 ± 1.0	72.5 ± 2.7	71.5 ± 1.0	0.244	0.069	0.902

Diet abbreviations are as follows: LP-LCL, low protein-low carbohydrate to lipid ratio; HP-LCL, high protein- low carbohydrate to lipid ratio; LP-HCL, low protein-high carbohydrate to lipid ratio; HP-HCL, high protein-high carbohydrate to lipid ratio.

The mucosal fold length (p<0.007) and thickness (p<0.038) significantly increased in fish fed on diets with low carbohydrate: lipid ratio, meanwhile enterocytes height and muscularis layer thickness were not affected by feeds (Table 4).

Table 4: Morphological changes in intestinal sections of *E. coioides* juvenile fed experimental diets for eight weeks. A different superscript in the same row denotes statistically significant differences (*p*<0.05).

Parameters	Treatments				Two-Way ANOVA		
	LP-LCL	HP-LCL	LP-HCL	HP-HCL	Protein	CHO/L	Protein × CHO/L
Mucosal fold length (µm)	528.5 ± 50.7[a]	535.7 ± 77.1[a]	422.9 ± 29.1[b]	428.4 ± 37.2[b]	0.836	0.007	0.979
Mucosal fold thickness (µm)	76.9 ± 5.9[a]	69.4 ± 4.8[b]	65.3 ± 7.6[c]	63.1 ± 6.6[c]	0.213	0.038	0.485
Enterocyte height (µm)	31.9 ± 2.2	27.3 ± 1.2	27.4 ± 1.6	26.6 ± 3.5	0.080	0.090	0.194
Muscularis thickness (µm)	49.5 ± 8.5	52.8 ± 11.9	35.5 ± 14.7	38.8 ± 1.4	0.595	0.05	0.994

Diet abbreviations are as follows: LP-LCL, low protein-low carbohydrate to lipid ratio; HP-LCL, high protein- low carbohydrate to lipid ratio; LP-HCL, low protein-high carbohydrate to lipid ratio; HP-HCL, high protein-high carbohydrate to lipid ratio.

Among hematological parameters only total hemoglobin (Hb) and hematocrit (Hct) percentage were significantly altered by experimental feeds (Table 5). In this sense, blood Hct percentage affected by dietary protein level (p<0.042) and carbohydrate: lipid ratio (p<0.001), whereas Hb content remarkably increased in fish fed on the high carbohydrate: lipid ratio diets (p<0.006).

Table 5: Hematological parameters (%, mean±SE, n=3) of *E. coioides* juvenile fed experimental diets for eight weeks. A different superscript in the same row denotes statistically significant differences (*p*<0.05).

Parameters	Treatments				Two-Way ANOVA		
	LP-LCL	HP-LCL	LP-HCL	HP-HCL	Protein	CHO/L	Protein × CHO/L
RBC ($\times 10^6$ µL^{-1})	1.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.0	0.384	0.051	0.248
WBC ($\times 10^3$ µL^{-1})	57.0 ± 7.9	54.2 ± 6.5	59.4 ± 6.5	56.4 ± 5.1	0.469	0.563	0.930
Hct (%)	30.6 ± 1.0^{a}	28.7 ± 1.8^{ab}	27.0 ± 1.3^{bc}	25.3 ± 0.8^{c}	0.042	0.001	0.711
Hb (g dL^{-1})	7.2 ± 1.6^{b}	6.9 ± 1.1^{b}	8.8 ± 0.1^{a}	8.7 ± 0.4^{a}	0.825	0.006	0.973
MCV (nm^3)	289.2 ± 36.1	318.3 ± 57.3	324.9 ± 29.5	297.9 ± 6.8	0.962	0.730	0.227
MCH (pg cell^{-1})	74.8 ± 13.0	97.6 ± 26.8	69.1 ± 59.9	102.9 ± 5.1	0.183	0.991	0.784
MCHC (g dL^{-1})	25.9 ± 3.7	30.3 ± 3.6	22.3 ± 19.4	34.5 ±1.6	0.191	0.956	0.521

Diet abbreviations are as follows: LP-LCL, low protein-low carbohydrate to lipid ratio; HP-LCL, high protein- low carbohydrate to lipid ratio; LP-HCL, low protein-high carbohydrate to lipid ratio; HP-HCL, high protein-high carbohydrate to lipid ratio. Abbreviations are as follow: RBC: red blood cells; WBC: white blood cells; Hct: hematocrit; Hb: hemoglobin, MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

The results of pancreatic enzymes activities revealed that total alkaline protease (Fig. 1a) increased in fish fed on the high protein content feeds and it was influenced by dietary protein content (*p*=0.001) but not carbohydrate: lipid ratio (*p*=0.19) or their interaction (*p*=0.47).

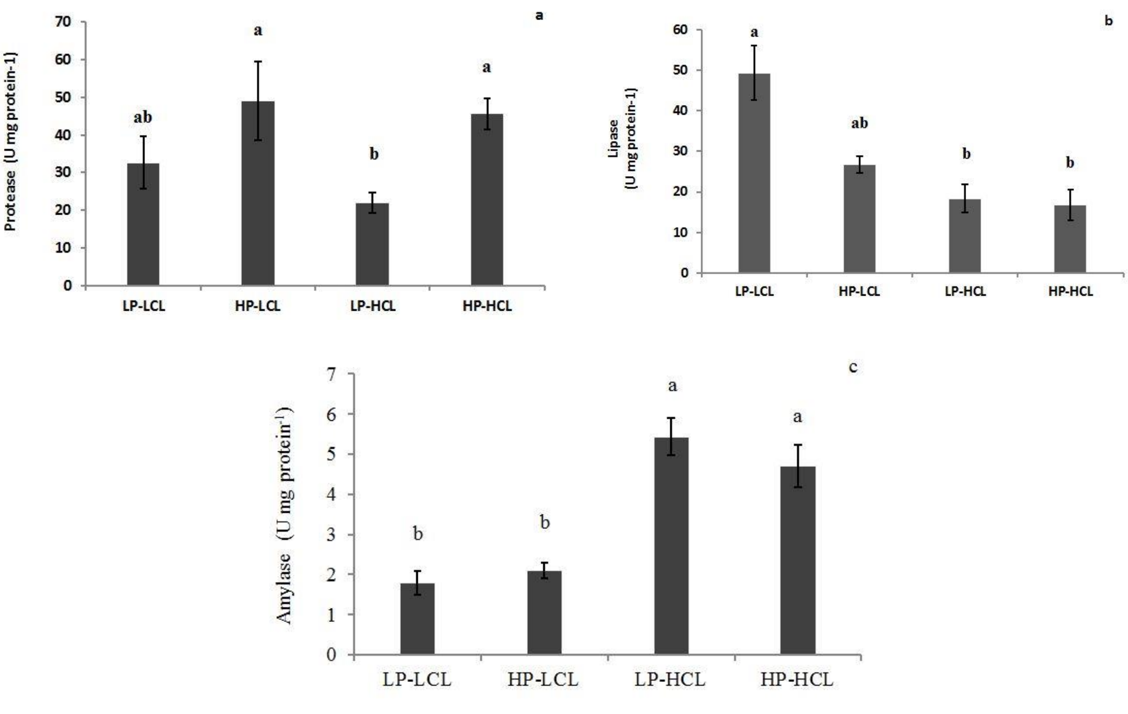

Figure 1: Digestive enzyme activity including protease (a), lipase (b) and amylase (c) in *E. coioides* juveniles fed experimental diets for eight weeks (mean±SE, n=3).

The bile-salt lipase activity (Fig. 1b) enhanced with reduction of dietary carbohydrate: lipid ratio and it was affected by dietary protein level (*p*=0.001), carbohydrate: lipid ratio (*p*=0.001) and their interaction (*p*<0.003). The activity of α-amylase (Fig. 1c) increased in fish fed on the high carbohydrate: lipid ratio diets and it was remarkably affected by dietary carbohydrate: lipid ratio (*p*=0.001) but not protein level (*p*=0.36) or their interaction (*p*=0.05).

Discussion

The findings of this study showed general health of fish especially survival rate was not compromised by drastic alternations in macronutrients in feed in a short-term period as previously described in other grouper species (Lupatsch and Kissil 2005; Li *et al.*, 2016; Gao *et al.*, 2018). Previous research in different grouper species revealed that these species need high protein (45–55%) requirement and prefer protein as energy source over carbohydrate and lipid (reviewed by Williams, 2009; Shapawi *et al.*, 2014). But, in the current study growth performance profoundly affected by dietary carbohydrate: lipid ratio rather than protein content. In this sense, results showed that fish fed on the low carbohydrate: lipid diets (1.4) had better growth and FCR than fish fed on the high carbohydrate: lipid ratio diets (2.9) indicating orange-spotted grouper is better adapted to utilize lipid compared to carbohydrate. In addition, fish fed on the LP-LCL diet showed protein sparing effect suggesting the sparing effect of lipid is superior compared to carbohydrate as also reported in European sea bass (Pérez *et al.*, 1997), brown-marbled grouper (*E. fuscoguttatus* Shapawi *et al.*, 2014), black-spotted croaker (*Nibea diacanthus*, Li *et al.*, 2017) and silvery-black porgy (*Sparidentex hasta*, Mozanzadeh *et al.*, 2017). Similarly, Wang *et al.* (2017) reported that dietary carbohydrate level above 13.5% may have negative effects on the growth performance of orange-spotted grouper. The appropriate dietary carbohydrate: lipid ratio in giant grouper (*E. lanceolatus*, Li and Wu, 2016) and hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂, Li *et al.*, 2019) juveniles were reported to be 0.8 and 1.25, respectively in high protein content diets (*Ca.* 50%). In contrast, Lin and Shiau (2003) reported that dietary carbohydrate: lipid ratios (0.78 to 7.23) did not affect growth rate and feed utilization in Malabar grouper (*E. malabaricus*) suggesting both NPES can be well utilized by this species. It should be mention these discrepancies in the outcomes of above-mentioned research related to different parameters such as fish species and its developmental stage, experimental condition mainly water temperature, feeding rate, molecular complexity of carbohydrate and also dietary carbohydrate and lipid contents that eventually affect carbohydrate matabolization (Kaushik and Médale 1994; Fountoulaki *et al.*, 2005).

In this study, FI remarkably influenced by dietary protein content and fish fed on the low protein content diets had greater FI possibly to meet their protein requirements for growth and metabolism. In this regard, it has been proved that amino acid-sensing systems significantly involve in the control of FI in fish by modulating the expression of anorexigenic (*e.g.* cholecystokinin and ghrelin) and orexigenic (*e.g.* agouti-related peptide and neuropeptide Y) hormones and neurotransmitters (Comesaña *et al*., 2018; Calo *et al*., 2021). Similar findings also described in other carnivorous fish like Malabar grouper (Tuan and Williams 2007), sharpsnout sea bream (*Diplodus puntazzo,* Coutinho *et al.,* 2012), golden pompano (*Trachinotus ovatus*, Wang *et al.,* 2013), brown-marbled grouper (Shapawi *et al.,* 2014) and black-spotted croaker (*Nibea diacanthus*, Li *et al.,* 2016). In addition, FCR remarkably increased in fish fed on the high carbohydrate: lipid ratio diets that could be attributed to poorer growth. In this sense, it has been revealed that increasing dietary carbohydrate: lipid ratio enhanced FCR in carnivorous fish species such as orange-spotted grouper (Wang *et al.,* 2017), Malabar grouper (Li *et al.,* 2016) and hybrid grouper (Gao *et al.,* 2018) due to low or moderate α-amylase activities in carnivorous species. Furthermore, high carbohydrate level in diet may negatively affects nutrients digestibility and digestive enzymes activities (Krogdahl *et al.,* 2005). Although no statistical differences were noticed in PPV and PER values between different treatments, fish fed on the lower carbohydrate: lipid ratio diets generally had higher PPV and PER values indicating higher protein sparing effects of L compared to carbohydrate as also reported in other groupers (Shapawi *et al.,* 2014; Rahimnejad *et al*., 2015; Li *et al.,* 2016; Gao *et al.,* 2018). In contrast, it has been reported that PER and FCR significantly increased with increasing dietary carbohydrate level in cobia (*Rachycentron canadum*) and has higher protein sparing effect compared to lipid (Ren *et al.,* 2011; Zhao *et al.,* 2020). In this study values of HSI and VSI elevated with increasing dietary carbohydrate: lipid ratio. Similar to our results, Liu *et al.* (2020) reported that increasing dietary carbohydrate: lipid ratio enhanced HSI and VSI in orange-spotted grouper might be due to increasing lipogenesis and glycogenesis that enhanced deposition of glycogen and lipid in the liver and visceral cavity of fish, respectively. In this sense it has been speculated that extra level of dietary carbohydrate would be accumulated as glycogen or lipid in the liver as previously observed in cobia (Ren *et al.,* 2011) and *Oplegnathus fasciatus* (Kim *et al.,* 2016), giant Grouper (Li *et al.,* 2016), red-spot grouper (*E. akaara,* Wang *et al.*, 2016), hybrid grouper (Gao *et al.*, 2018) and orange-spotted grouper (Wang *et al.,* 2017; Liu *et al.,* 2020).

Our findings demonstrated whole-body lipid content increased in fish fed on the low carbohydrate: lipid diets indicated excess lipid intake would induce a lipid deposition in whole body as reported for other carnivorous such as Malabar grouper (Lin and Shiau 2003); humpback grouper (*Cromileptes altivelis,* Usman *et al.,* 2005); orange-spotted grouper (Cheng *et al.*, 2006; Wang *et al.*, 2016); Giant Grouper (Li *et al.,* 2016) and hybrid grouper

(Rahimnejad *et al*., 2015; Gao *et al*., 2018; Kian *et al.*, 2019). These results demonstrated that the lipogenesis is mainly regulated by the lipid intake rather than the glycolytic pathway in carnivorous fish species (Dias *et al*., 2004). In addition, fish have low ability to utilize carbohydrate. This mean, they gain less energy when use high carbohydrate diet, compared to the same energy by fat. So, higher fat in fish body in low CHO: L is due to higher metabolized energy that was deposited in the tissues.

High levels of carbohydrate and/or non-starch polysaccharides in diet may be responsible for negative impacts on growth, appropriate functional of organs and animal health especially in marine carnivorous fish species (Gatlin *et al.,* 2007; Enes *et al.,* 2009). Histopathological changes in the intestine may vary depending on the species and dietary composition used in the experiments. In this study, fish fed low carbohydrate: lipid ratio diets had greater mucosal fold length and thickness than fish fed on the high carbohydrate: lipid ratio that might be attributed to the occurrence of lipid steatosis in these groups. Similarly, it has been reported that increasing dietary lipid level induced drastic histomorphological changes including enlargement of enterocytes, epithelial cells and supranuclear vacuoles of enterocytes due to steatosis and accumulation of numerous L droplets in the enterocyte supranuclear vacuoles of pikeperch (*Sander lucioperca*, Kowalska *et al.,* 2011) and rainbow trout (*Oncorhynchus mykiss*, Trenzado *et al.,* 2018). In the present study the values of Hct was reduced in fish fed on the low carbohydrate: lipid ratio diets that was coincided with the reduction of RBC in these groups. In fact, the RBC count decreased with increasing dietary lipid, that suggesting oxidative stress that may be resulted in RBC cytoplasm lysis in this group. In addition, the Hb content increased with in fish fed on the high carbohydrate: lipid ratio diets that may indicate increased metabolic burden in these groups. These results suggesting that increasing Hb level in fish fed on the high carbohydrate: lipid ratio diet may be compensated Hct and RBC reduction in these groups. In contrast, Li *et al.* (2012) reported that increasing dietary carbohydrate: lipid ratio significantly reduced RBC and Hb in blunt snout bream, *Megalobrama amblycephala*. On the other hand, Mozanzadeh *et al.* (2017) reported that RBC count increased in silvery-black porgy fed on high carbohydrate: lipid diets, but RBC count gradually reduced with increasing carbohydrate: lipid ration in yellow catfish (*Pelteobagrus fulvidraco*, Wang *et al.,* 2014). The discrepancies between abovementioned results may be related to fish species, their developmental stage and health status, experimental trial condition and feed composition.

The digestive enzymes capacity of fish in response to a dietary composition determines the efficacy of a given diet (Perez-Jimenez *et al.,* 2009). In the present study, the response of digestive enzymes activities was correlated with the amount of their substrates in the diets. In this sense, the specific activity of proteases was increased in fish that fed on the high protein content diets as previously demonstrated in gilthead seabream (*Sparus aurata*, Fountoulaki *et al.,* 2005) and European sea bass (García-Meilán *et al.,* 2016). In addition, bile-salt lipase activity was enhanced in fish that fed on the low carbohydrate: lipid diets mainly due to high lipid content in these feeds. Previous studies also demonstrated lipase activity remarkably increased in response to augmentation of dietary lipid level in blunt snout bream (*Megalobrama amblycephala*, Li *et al.,* 2012) and large yellow croaker (*Larmichthys crocea*, Zhou *et al.,* 2016). In addition, bile-salt lipase activity also affected by dietary protein level as also reported in European seabass (García-Meilán *et al.,* 2016). In this sense, Murashita *et al.* (2008) reported that lipase gene expression was up-regulated following an increase in dietary protein content in yellowtail (*Seriola quinqueradiata*). Furthermore, the activity of α-amylase was significantly increased in fish fed on the high carbohydrate: lipid ratio diets as also reported in different carnivorous fish species fed on high carbohydrate content diets (Lundstedt *et al.,* 2004; Fountoulaki *et al.,* 2005; Ren *et al.,* 2011; Zhou *et al.,* 2016; Gao *et al.,* 2018; Liu *et al.,* 2020). These results indicated that the digestive enzyme capacity of orange-spotted grouper is remarkably elastic in response to the amounts of macronutrients in a diet.

In conclusion, the findings of this research demonstrated protein sparing effect of dietary lipid in orange-spotted grouper. In addition, growth performance was compromised in fish fed on high carbohydrate diet indicating this species could not utilize high amount of carbohydrate properly compared to dietary lipid. Also, increasing dietary carbohydrate: lipid ratio remarkably enhanced HSI in fish may due to glycogen deposition in the liver. Furthermore, reduction of dietary carbohydrate: lipid ratio resulted in lipid deposition in the whole body of fish and increased gut's mucosal length and thickness may due to lipid steatosis in the enterocytes. The response of the digestive enzymes activities in this species were closely correlated with the amount of their substrates in the diets. According to the results dietary lipid is more preferable than carbohydrate as an energy source and induces protein sparing effect in a low protein content diet.

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