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The Use of Carotene Materials as the Source of Red Color Pigmentation on Leopard Grouper Larvae (*Plectropomus leopardus*)

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Abstract

Daniar Kusumawati and Ketut Maha Setiawati. 2016. The Use of Carotene Materials as the Source of Red Color Pigmentation on Leopard Grouper Larvae (*Plectropomus leopardus*). *Aquacultura Indonesiana. 17 (2): 35-45*. Carotenoid is one of nutrition factors which can improve red color pigmentation. The purpose of this study was to investigate the best type of carotene which can increase red pigment performance and to evaluate how long the effect should be retaining in order to maintain red color performance on leopard grouper seeds. This study consisted of 2 experiments; those were the effect of various kinds of carotene materials in feed addition and the effect of the termination of the addition of carotene materials in the feed toward red color performance on leopard grouper seed. The treatments given to the first experiment were the provision of various kinds of carotene materials which were type of A. *Haematococcus pluvialis*, B. *Phaffiarhodozyma*, C. Astax Oil, D. Control (without carotene materials) and the treatments to the second experiment are A. Leopard grouper seed – phaffia was still given additional *Phaffia rodhozyma*, B. Leopard grouper seed – haematococcus was still given additional *Haematococcus pluvialis*, C. leopard grouper seed (from larvae - phaffia / *haematococcus*) was terminated from carotene materials addition. The first experiment was applied from larva up to D60 seed and for the second experiment was the follow-up response from the first experiment conducted on D60 seed up to D150. Based on the study result, it indicates that carotene materials of *Haematococcus pluvialis* type gave the best color performance improvement (P value 4.71 x 10^{-7} < 0.05). The provision of carotene materials intake decreases color performance both visually and total contain of carotene (P value 5.97 x 10^{-5}< 0.05) (Tabel 1). Carotenenoids should be continuously given as trigger to maintain red color performance on leopard grouper seed. There was protein band in the range of 82.1 – 84.2 kDa which was assumed as protein expression of astaxanthin.

Keywords : Carotenoid; Leopard grouper; Pigment; Red color

Introduction

There are two species of coral trout *Plectropomus leopardus* (leopard grouper) and *Plectropomus maculates* (barred cheek corral trout). Leopard grouper have higher economical value than barred cheek corral trout and the most popular than others grouper due to the red colour that its have. Ther red colour is symbol of luck for the hongkong community which is the largest importer of grouper. Leopard grouper fish (*Plectropomus leopardus*) have red color pattern with blue dots spread all over the body. Pigment development has occurred in embryo and continues to develop along with the growing size of the fish. During intensive breeding from larva up to consumed size is found that red color of leopard grouper fish fades. This is extremely different when it is compared with red color performance on leopard grouper fish from fishing in nature which looks so bright. Leopard grouper fish in nature have bright red color pigment without melanocytes indication with clear blue dots.

Fish color pattern has very important role in partner selection, spawning indicator, camouflage means, sun protection, thermoregulation (Karlsson, 2001; Korzan *et al.*, 2008) and is also the main factor determining the high quality of the fish (Gouveia *et al.*, 2003). Skin color performance is determined by pigment cells on integument tissue (Nery and Castrucci, 1997). Pigment cells or chromatophore which is neural crest derivate are categorized based on pigment color produced and its internal structures. Chromatophore can perform translocation centrifugally by way of granule pigment moves in dispersion and centripetal translocation in which granule movement is focused (aggregation) into perinuclear zone (Nery and Castrucci, 1997). The translocation process will affect pattern change and color change on skin. Pigment cells perform translocation due to response toward environment change like temperature variation, light intensity, surrounding environmental condition, food nutrition (Gouveia *et al*., 2003; Yasir and Qin, 2010), and genetic (Kelsh, 2004; Sugie *et al*., 2004) which are captured in the form of specific

signals received by neuron cells and hormone in blood flow to be captured by specific receptor in pigment cells surface (Nery and Castrucci, 1997; Amiya *et al*., 2008). Color variation on skin is determined by position and the number of pigment cells on skin. However, mechanism on how color pattern performance of a species can be formed has not yet found (Sugie *et al.*, 2004).

Factors that will be studied was food nutrition factor in which it has been found out that carotenoids are the materials responsible in red, orange, and yellow pigment formation on fish and prawn (Packer, 1992). Carotenoids are only able to be synthesized by photosynthetic organism which are algae, plants and bacteria as well as some non-photosynthetic organisms which are bacteria and fungi (Hirschberg, 1999). So carotenoid needs on animals especially fish is obtained from nutrition intake added in the feed. Upon intake, fish can modify alimentary carotenoids and store them in integument system and other tissues. Therefore, study on the use of carotene materials as means to increase red color performance on leopard grouper fish needs to be conducted.

Materials and Method

This study consists of 2 experiments those were (1) The effect of carotene materials adding in food toward leopard grouper fish larva and (2) The effect of carotene materials adding on food toward seed of carotene materials enrichment result.

Experiment I: The effect of carotene materials adding in food toward Leopard grouper larvae

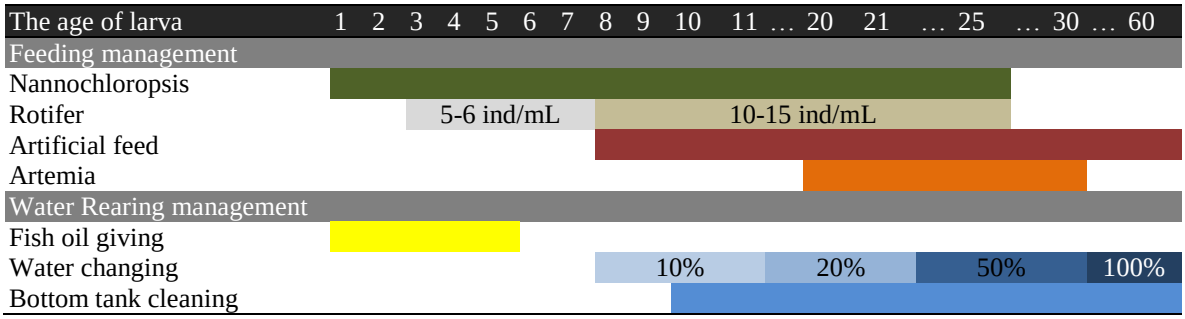
Treatment

The treatment tested was the giving of different types of carotene materials which were A. *Haematococcus pluvialis* (NatuRose), B. *Phaffia rhodozyma* (Red Yeast Phaffia), C. Astax Oil, and D. without carotene materials adding (control). The application of carotene materials adding was performed on rotifer (fish larva with the age of D3 – D30) and artificial feed (fish larva with the age of > D30). Carotene materials additional dose on rotifer by 0.024 g/L rotifer of *haematococcus* and phaffia (solid material) and dose on rotifer by 0.25 mL/L rotifer of astax oil (liquid material) with rotifer stock density ranges from 20-40 ind/mL. The length of enrichment on rotifer was 5-6 hours. Carotene materials adding in artificial feed of solid kind was 0.01 g/g feed and of liquid kind was 1 mL/g feed. Carotene materials adding in solid kind on artificial feed (pellet) was performed by mixing carotene materials with cmc (binder) with the comparison of 3:2 then mixed well with the artificial feed then sprayed with water until it was attached and then dried. Enrichment of carotene materials on liquid kind of artificial feed only needs to be performed by mixing directly with the feed well then dried.

Larva Rearing

Leopard grouper fish were preserved in tank with capacity of 6 tons with the size of 2.75 x 1.75 x 1.5 m. Egg of leopard grouper grouper were spread with density of 10 eggs/L, the larvae then rearing until juvenile or days 30 (D30) with 3 times repetition. Breeding management follows good fish breeding method and the larva rearing was according to Sugama *et al* (2000). Feeding for leopard grouper fish follows feeding management (Table 1) with feeding frequency of twice a day (morning and afternoon) for natural feed (rotifer and artemia) kind while artificial feeds were given 4-6 times a day. The initial feed that will be used was rotifer which was given to D2 in the afternoon. Meanwhile, artemia feeding would be started on D20. Water changing was started along with pellet giving as the initial. Bottom vessel cleaning would be started when larva were in the age of D10. Water changing would be performed gradually in accordance to larva development (Table 1).

Table 1. Feeding management of leopard grouper (*Plectropomus leopardus*) larvae



Experiment II: The effect of carotene materials adding on food toward seed of carotene materials enrichment result

Treatment

The seeds used were in the age of D60 from the study result of activity I in phaffia and *haematococcus* treatment with total average of length size of 3.63 $\pm$ 0.22 cm. The treatments tested were A. leopard grouper grouper fish seed – *haematococcus* was still given additional *Phaffia rhodozyma*, B. leopard grouper grouper fish seed – *haematococcus* was still given additional *Haematococcus pluvialis*, C. leopard grouper fish seed – phaffia/*haematococcus* without carotene materials adding (carotene materials adding termination) with three replication. Dose and application of carotene materials adding on artificial feed follows activity I method.

Seed rearing

Seeds in the age of D60 were reared in polycarbonate vessel with the capacity of 300 L with spread density of 100 fish with flow throw water system. Rearing was performed for 90 days. Feeding was performed in ad libithum with frequency of twice a day. Syphoning was performed in the morning before feeding.

Parameter

Parameter observed in this study was larvae development involving total length, standard length, dorsal spine and anal spine length for 30 days with monitoring interval of 5 days. The last sampling was performed on D60 to measure the growth of larvae in body length and weight and total carotene absorbed. Seed skin protein of Leopard grouper grouper fish profile in the age of D60 and D150 were respectively analyzed using SDS PAGE with separating gel concentration of 12.5% and stacking gel 5%. Running sample was performed with constant flow of 30 mA for more or less by 60 minute and staining with commasie brilliant blue to determining protein band.

Data Analysis

Descriptive analysis was conducted for the data from experiment I and II, then followed by statistical test with ANOVA using epitools (free application from *epitools.ausvet.com.au*) and significant test with LSD test (Least Significant Difference). Protein profile data obtained was analyzed descriptively by comparing protein profile on each treatment.

Results and Discussion

Experiment I: The effect of carotene materials adding in food toward leopard grouper fish larva

Larva(e) development

Based on study result, it was found that the total length of larva development on each treatment for rising time of 30 days statistically has significant difference (P value $2.56 \times 10^{-4} < 0.05$) (Table 2). The highest length growth exists on carotene feeding treatment of phaffia kind then followed by *haematococcus* kind, astax oil and control treatment.

Seeing larva morphology development pattern on each treatment, it has different pattern (Figure 1). The growth of total length and standard length of all treatment on D5 up to D10 has the same growth pattern (Figure 1^A – 1^B). However entering D30, it is seen that phaffia treatment has high total and standard length growth followed by *haematococcus*, astax oil and control. On dorsal and ventral spine growth, all treatments give varied pattern (Figure 1^C – 1^D). Dorsal and anal spain (spine) growth starts to occur on D10. Dorsal and anal spine length adding continues to experience an increase and entering D25, there is different pattern in which on phaffia and *haematococcus* treatment, dorsal and anal spine length experiences shortening instead of growth. Meanwhile, on astax oil and control treatment, the growth of dorsal and anal length continues to occur. This indicates that phaffia and *haematococcus* giving treatment, larva metamorphose development enters juvenile phase far more rapidly. It is indicated by the shortening of dorsal spine.

Entering juvenile phase (D60) it is found that length growth on each treatment has significantly difference (P value $4.81 \times 10^{-8} < 0.05$) (Table 3). The highest length growth lies on carotene materials giving treatment of phaffia kind then followed by *haematococcus*, astax oil and control treatments. Meanwhile, weight increase on juvenile leopard grouper fish of each treatment has significant difference (P value $7.5 \times 10^{-9} < 0.05$) (Tabel 3). The highest weight adding lays on carotene materials giving treatment of phaffia kind then followed by *haematococcus*, astax oil and control treatments.

Table 2. Total length growth of Leopard grouper grouper fish larva of D30 on each treatment

	TL D30 (mm)		Length growth rate (%/day)	
Treatment	**Mean**	**SD**	**Mean**	**SD**
Astax Oil	12.66 b	1.27	42.4 b	4.23
Haematococcus	14.94 a	0.71	49.8 a	2.35
Phaffia	17.2 a	0.57	57.33 a	1.89
Control	12.22 c	2.56	40.73 c	8.53

*Notation with different letter shows significantly difference

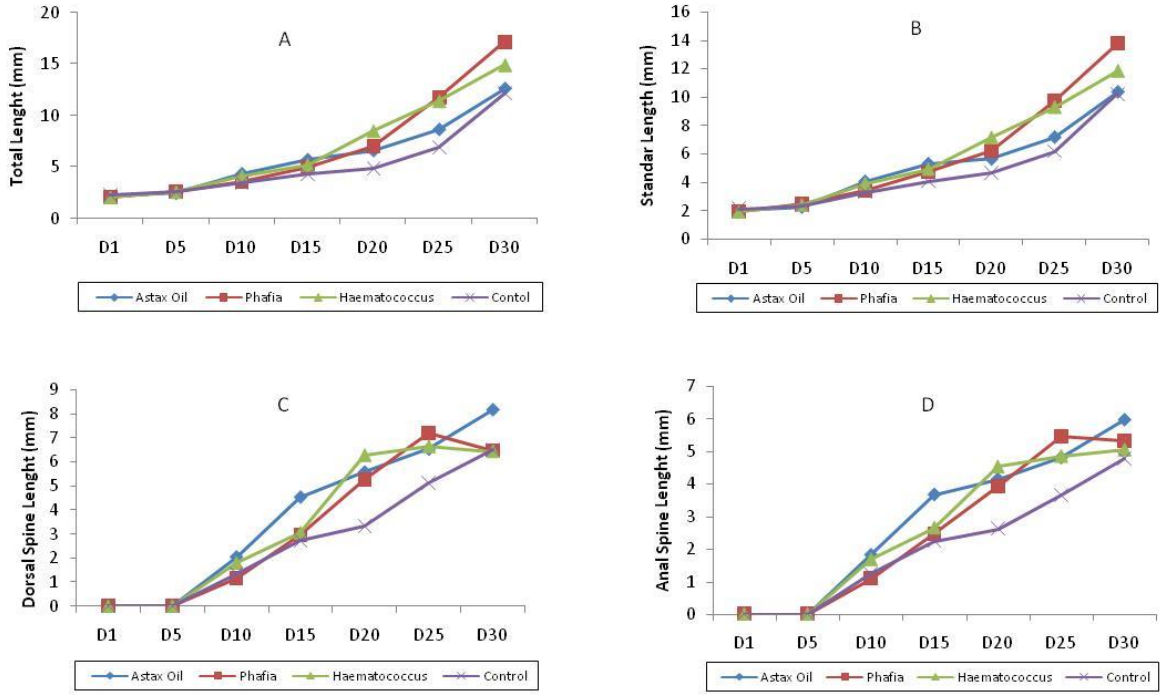


Figure 1. Larva morphology development pattern. Total length growth (A), standard length (B), dorsal spine length (C), anal spine length (D).

Table 3. The growth of total length and weight of leopard grouper fish of D60 on each treatment

Treatment	Total Length (mm)		Length growth rate (%/day)		Total Weight (g)		Weight growth rate (%/day)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Astax Oil	3.44 b	0.13	5.73b	0.22	0.48 c	0.06	0.8 c	0.1
Haematococcus	3.69a	0.12	6.15 a	0.2	0.8 b	0.07	1.33 b	0.12
Phaffia	3.76a	0.39	6.27a	0.66	0.87 a	0.29	1.45 a	0.48
Control	2.95 c	0.26	4.92 c	0.43	0.35 d	0.09	0.59 d	0.15

*Notation with different letter shows significant difference

The effect of carotene materials giving on Leopard grouper grouper fish seeds

Based on the result of rising for 60 days, color performance on leopard grouper fish seeds given additional carotene materials visually starts showing betterment (Figure 2). This is indicated by total carotene contain in leopard grouper fish body which increase compares to control in which total carotene of each treatment shows significant difference (P value 4.71 x 10^{-7}< 0.05) (Figure 2). The highest total carotene lays on *haematococcus* adding treatment (124.92 µ/g) followed by phaffia adding treatment (81.86 µ/g), astax oil (70.96 µ/g) and control (48.25 µ/g).

The adding of carotene materials of *haematococcus* kind gives better red color betterment compared to other treatments with reddish orange color starting from dorsal, anal, caudal fins up to all over the body (Figure 3). Meanwhile, in carotene materials adding treatment of phaffia kind give orange up to reddish orange performance on starting from dorsal, anal, caudal fins up to all over the body

66.7% which is dominated by reddish orange color on dorsal and anal fin part and orange color on caudal fin and body. Meanwhile, astax oil adding treatment gives color performance with uneven spread proportion starting from brownish yellow up to orange. On dorsal and anal fins, color proportion is 66.7% which is dominated by light yellow color and 33.3% of brownish yellow. On caudal part, color proportion is 66.7% which is dominated by light yellow and 33.3% orange. And light yellow on all body part. In control treatment, color performance appears starts from brownish yellow up to orange in which all part of body is dominated by brownish yellow and on caudal, dorsal and anal fin parts, the color proportion is 66.7% which is dominated by brownish yellow and 33.3% orange.

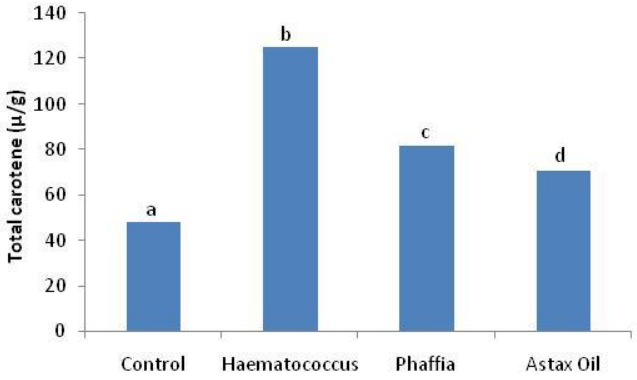


Figure 2. Total carotene contain in Leopard grouper grouper fish seeds of D60

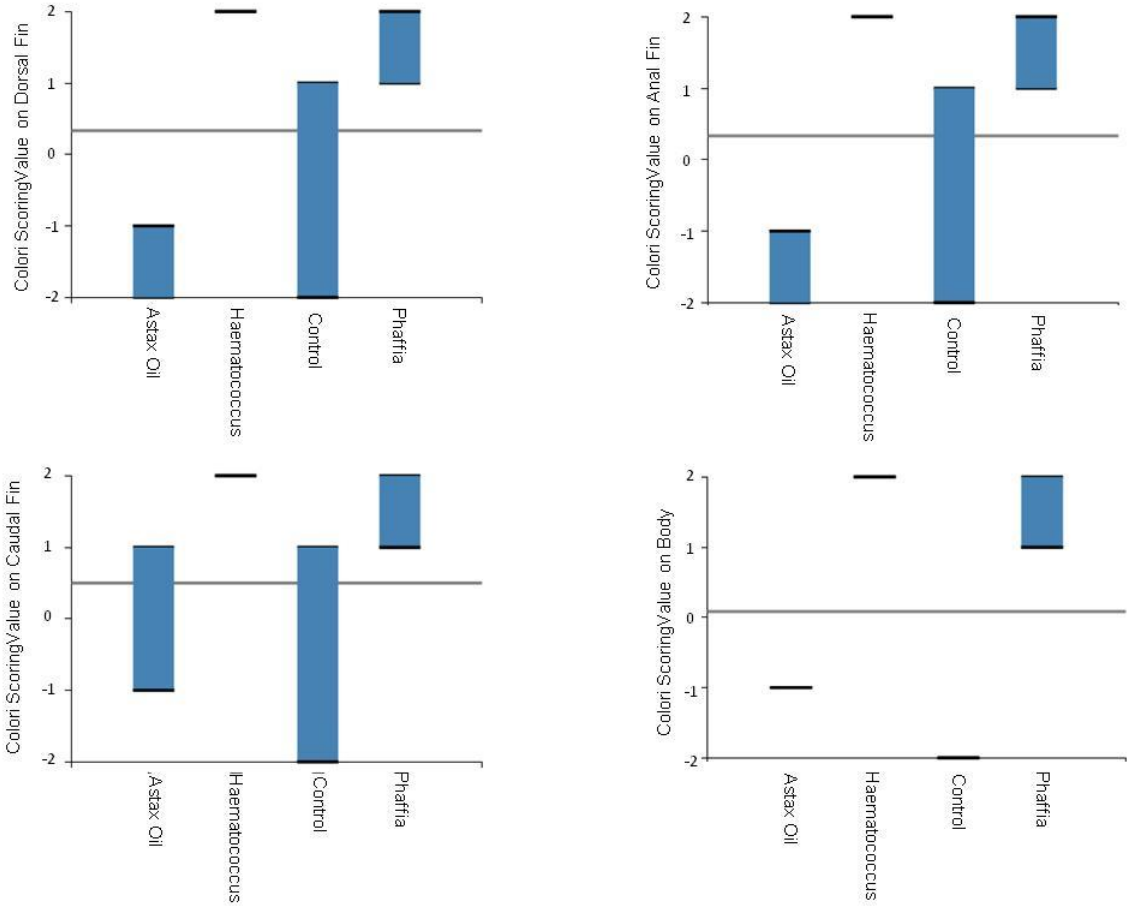


Figure 3. Visual color scoring value on seeds of D60. Description: -3 =brown; -2 =brownish yellow; -1 =light yellow (yellowish); 0 =yellow; 1=orange; 2 =reddish orange; 3 = red

Skin protein profile

Based on skin tissue protein profile confirmation, it is found that each treatment gives varied allele confirmation. Protein profile variation is indicated by appearance and disappearance of protein band of skin organ (Figure 4). In control treatment, 11 alleles appear with 1 specific allele and there are 2 allele proteins lost, astax oil treatment 17 alleles appears with 3 specific alleles, phaffia treatment shows 18 alleles with 1 specific allele and there is 1 protein allele lost, *haematococcus* treatment shows 17 alleles with 1 specific allele (Table 4). In Leopard grouper grouper fish of D60, protein band confirmation can show specific band expressing treatment given. In control treatment there is specific protein band on molecule weight of 22kDa and there is protein band lost on molecule weight of 29.4 kDa and 22.7 kDa. Meanwhile in astax oil treatment, there is specific protein band on molecule weight 85.4 kDa, 52.1 kDa, 32.2kDa. In phaffia treatment there is specific protein band with molecule weight of 105.3 kDa and there is protein band lost on molecule weight of 109.4 kDa. And in *haematococcus* treatment there is specific protein on molecule weight of 23 kDa.

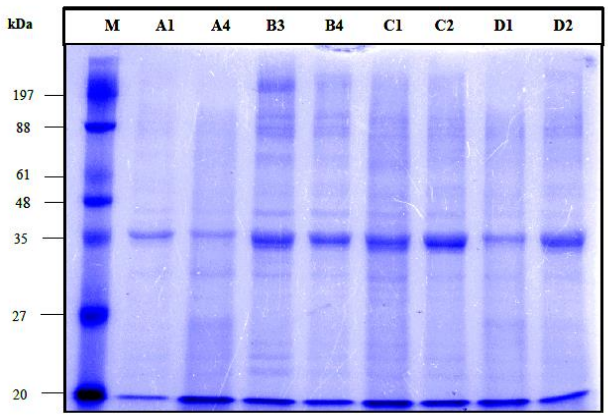


Figure 4. Skin protein profile of leopard grouper fish of D6. Description: A1 - A4 = control, B3 – B4 = astaxOil, C1 –C2 = *Phaffia rodhozyma*, D1 – D2 = *Haematococcus pluvialis*

Table 4. Variation of skin protein molecule of leopard grouper fish seeds of D60 in each treatment

Protein band	Treatment							
(kDa)	K1	K4	A3	A4	P1	P2	H1	H2
197	-	-	+	+	+	+	-	-
109.4	+	+	+	+	-	-	+	+
105.3	-	-	-	-	+	+	-	-
88	+	+	+	+	+	+	+	+
85.4	-	-	+	+	-	-	-	-
84.2	-	-	-	-	+	+	+	+
68.5	+	+	+	+	-	+	+	+
67.7	-	-	-	-	+	-	-	-
52.1	-	-	+	+	-	-	-	-
51.6	-	-	-	-	-	+	-	+
50.9	+	+	+	-	+	+	+	+
44.5	+	+	+	+	+	+	+	+
42.8	+	+	+	+	+	+	+	+
36.1	+	+	-	-	-	-	+	
35.7	-	-	+	+	+	+	-	+
32.2	-	-	+	+	-	-	-	-
30.9	+	+	+	+	+	+	+	+
29.4	-	-	+	+	+	+	+	+
28	-	+	+	+	+	+	+	+
26	-	+	-	-	-	-	+	+
24.4	-	-	+	+	+	-	-	-
24.2	-	-	-	-		+	+	+
23.2	-	-	+	+	+	+	-	-
23	-	-	-	-	-	-	+	+
22.7	-	-	+	+	+	+	+	+
22	+	+	-	-	-	-	-	-

Description:
+ : positif
- : negative

Experiment II:The effect of carotene materials enrichment termination on seeds of genrichment result

Seeds growth

Based on Rising for 3 months (D150) it is known that length growth rate on each treatment is not different significantly (P value 0.06>0.05) while weight adding (%/hour) has significant value (P value 0.01<0.05) (Table 5). The highest length growth and weight adding rate on carotene materials adding treatment of *haematococcus* kind, followed by phaffia then control. *Haematococcus* adding treatment gives average length adding rate of 7.4%/day and average weight adding rate of 0.9%/day. Then in phaffia giving treatment gives average length growth rate of 6.8%/day and average weight adding rate of 0.8%/day. Meanwhile, control treatment gives average length growth rate of 6.5%/day and average weight adding rate of 0.7%/day.

The effect of carotene materials adding termination toward color performance

During 3 months, it is seen that leopard grouper fish with carotene and those without carotene have varied red color performance (Figure 7). Obviously, the older the age and the bigger the size, pigmentation pattern starts to create more firmly and clearly. In phaffia giving treatment, by 80% leopard grouper fish seeds on dorsal and anal fin have orange color pattern, while the rest have brownish yellow (10%) and yellow (10%) color. On caudal fin part, 80% is dominated by reddish orange, while the rest have brownish yellow (10%) and orange (10%) color pattern. On body part, 90% is dominated by orange and 10% brown color (Figure 5).

In *haematococcus* treatment, 50% leopard grouper fish seeds have reddish orange color on dorsal fin part and 40% is orange while the rest is yellow. On anal fin, 60% seeds have reddish color and 40% is orange. On caudal fin part, 50% seeds have reddish orange color domination and 50% is orange. On body part, 60% seeds have orange color domination and 40% have reddish orange color domination (Figure 5).

On the seeds that carotene materials giving is terminated, red color pigmentation decrease appears on all over the body. On dorsal fin part, 40% seeds have brownish yellow color domination, 30% is orange, 10% is yellow, 10% is reddish orange and 10% is brown. On anal fin part, 50% seeds have brownish yellow color domination, 30% is orange, 10% is reddish orange and 10% brown. On caudal fin part, 70% seeds have reddish orange color domination and 30% is orange. On all over the body of seeds (100%) have brownish yellow color domination (Figure 5).

Table 5. Length growth rate and weight growth rate of leopard grouper fish seeds on each treatment

Treatment	Length growth rate (%/day)			Weight growth rate (%/day)		
	Mean		**SD**	**Mean**		**SD**
Haematococcus	7.4	a	0.93	15.87	b	4.1
Phaffia	6.83	a	0.81	13.42	a	2.7
Carotene giving termination	6.49	a	0.73	11.03	ab	3.24

* Notation with different letter shows significant difference

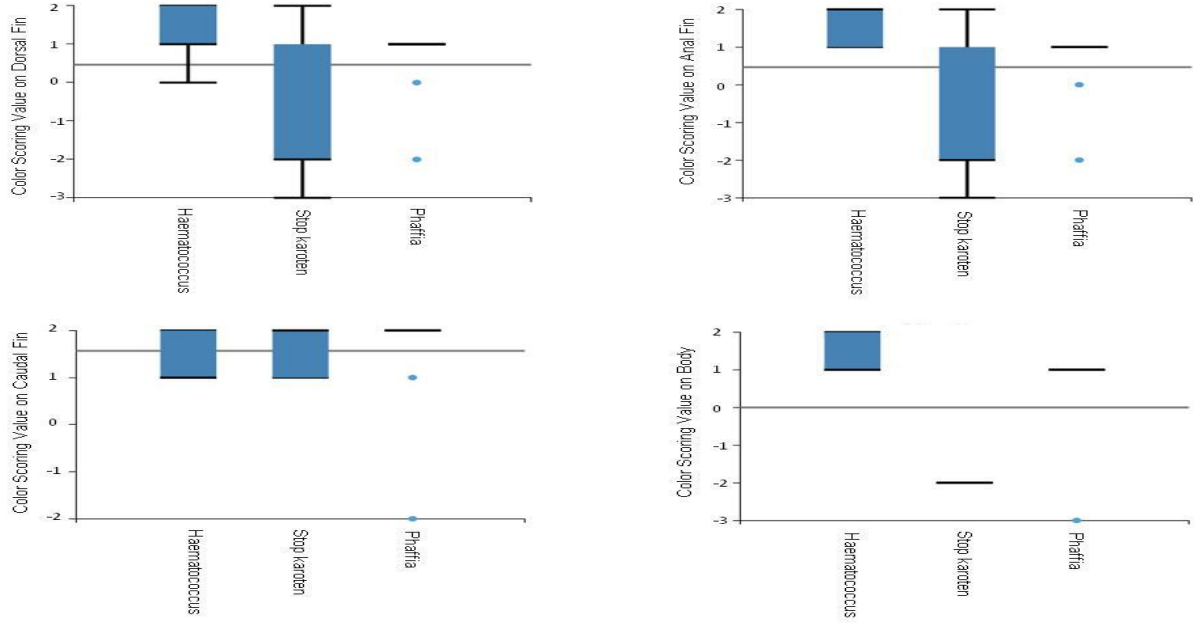


Figure 5. Visual color scoring value on seeds of D150. Description: -3 = brown; -2 =brownish yellow; -1 = light yellow (yellowish); 0 = yellow; 1= orange; 2 = reddish orange; 3 = red

Based on analysis result on total carotene, it is found that termination of carotene materials adding gives significant difference toward the total carotene contain (P value 5.97 x 10^{-5} < 0.05). The highest total carotene contain lays on *haematococcus* adding treatment (108.52 µ/g) followed by phaffia adding treatment (81.86 µ/g), and carotene termination (69.54 µ/g) (Figure 6).

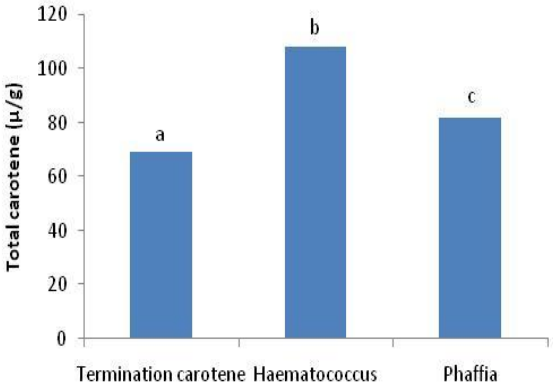


Figure 6. Total caroteneon leopard grouper fish seed sin the age of D150

Skin protein profile

Based on skin tissue protein profile confirmation of juvenile of D150, it shows varied allele confirmation and is different from juvenile skin protein profile (Figure 7). In carotene termination treatment, 20 alleles with 4 specific proteins appear and there is 1 protein allele lost (24 kDa), while in phaffia and *haematococcus* treatments there is no specific protein appears (Table 6).

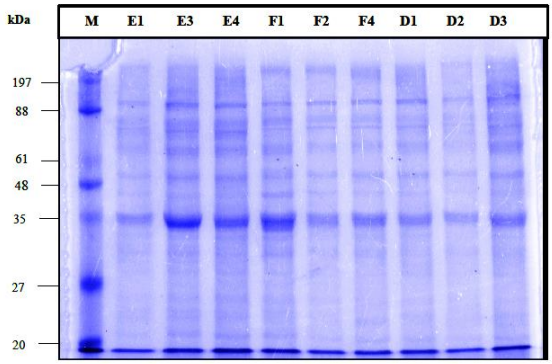


Figure 7. Skin protein profile of leopard grouper fish seeds of D60. Description: E1 - E4 = carotene termination, F1 – F4 = *Phaffia rodhozyma*, D1-D2 = *Haematococcus pluvialis*

Table 6. Variation of skin protein molecule weight of Leopard grouper grouper fish seeds of D150 on each treatment

Protein band	Treatment								
(kDa)	E1	E3	E4	F1	F2	F4	D1	D2	D3
197	-	+	+	-	-	-	-	-	-
171.4	-	-	-	-	-	-	-	-	+
151.3	+	+	+	-	-	-	-	-	-
108.4	+	+	+	+	+	+	+	+	+
82.1	-	-	-	+	+	+	+	+	+
81.4	+	+	+	-	-	-	-	-	-
76.9	+	-	-	+	+	+	+	+	+
75.8	-	+	+	-	-	-	-	-	-
66.3	+	-	-	+	+	+	+	+	+
65.4	-	+	+	-	-	-	-	-	-
52.3	+	+	+	+	+	+	+	+	+
44.2	-	-	-	-	+	+	+	+	+
43.6	+	+	+	+	-	-	-	-	-
34.9	+	-	-	-	+	+	+	+	+
34.3	-	+	+	+	-	-	-	-	-
32.4	+	+	+	-	-	-	-	-	-
30.4	+	+	+	+	+	+	+	+	+
29.5	+	+	+	-	-	-	+	-	-
28.3	+	+	+	+	+	+	+	+	+
25.2	+	+	+	+	+	+	-	-	+
24.8	-	-	-	+	-	-	-	-	-
24	+	+	+	-	-	-	-	-	-
23.6	+	+	+	-	-	-	+	-	-
23.3	-	-	-	+	-	-	-	-	-
22.6	-	-	-	-	-	+	-	+	+
20.8	+	+	+	+	+	+	+	+	+

Description:
+ : positif
- : negative

Discussion

The giving of carotene materials on phaffia and *haematococcus* kinds on larva provides better length and weight growth rate compares to control treatment. Carotene materials as additional materials in seed's feed rations are highly necessary to drive larva and seeds development. The giving of carotene materials of *haematococcus* kind can drive yellow croaker (*Pseudos ciaenacrocea*) (Li *et al.*, 2014) and rainbow trout fish (Sheikhzadeh *et al.*, 2012) fish. *Haematococcus* have high lipid contain as to be used as energy supply for fish to stimulate its development. Carotene materials and phaffia kind can stimulate salmon fish growth as well (Sanderson and Jolly, 1994). Phaffia is yeast that has excellent nutrition contain which are protein by 22%, fat by 23% and fiber by 3%.

The use of carotene materials as additional materials in enriching natural feed and artificial feed can increase red color performance improvement on leopard grouper fish larva. From various kinds of carotene materials used, carotene materials of yeast kind which is *Phaffia rhodozyma* and algae which is *Haematococcus pluvialis* give significant increase on red color performance when it is seen visually. Seeds D60 performance on both phaffia and *haematococcus* treatment give united pattern on body, dorsal, anal and caudal fins. Red color performance on leopard grouper fish of D60 using carotene materials of phaffia kind has orange and reddish orange color domination on body, while on seeds which give carotene materials of *haematococcus* kind has even color performance which reddish orange. However, along with the length of rising, color performance which is given also develops. On seeds of D150, it is seen that color performance given on phaffiaadding gives orange color on body part and orange up to reddish orange colors on fin part. Meanwhile, in *haematococcus* adding treatment, color performance given on body and fin parts is orange up to reddish orange colors but there is 1 fish sample with yellow caudal fin (Table 7).

Table 7. Deposition of pigmentation on Leopard grouper grouper fish seed based on visual color scoring

Seeds age	Treatment	Fin			Body
		Dorsal	Anal	Caudal	
D60	*Haematococcus*	2	2	2	2
	Phaffia	1-2	1-2	1-2	1-2
D150	*Haematococcus*	0-2	0-2	0-2	0-2
	Phaffia	1	1	1	1

It is seen that color reduction occurs on seeds of D150 compares to when they are still in D60. This color reduction can occur due to the low of fish ability in digesting and absorbing carotene (Table 8). This indicates that along with the age adding, carotene materials absorption accumulation is not increasing. In phaffia treatment, carotene accumulation in the age of D60 up to D150 is consistent; meanwhile in *haematococcus* treatment tends to decrease by 13.13%. And by seeing on total contain of carotene on carotene materials kind, the most efficient total absorption of carotene is in phaffia treatment by 4.15% while in *haematococcus* treatment is only 0.49%.

Table 8. Total accumulation of carotene on seeds and materials

Treatment	Total Carotene (µg/g)		
	Seeds of D60	Seeds of D150	Carotene materials
Haematococcus	124.92	108.52	25366.68
Phaffia	81.86	81.86	1970.78

Carotenoid is a kind of material which is hydrophobic which is difficult to dissolve in water environment but easily dissolve in fat. Based on the explanation, it is found that lipid or fat level in feed can give positive effect in carotene dissolution as to ease absorption in the body (Nickell and Bromage, 1998; Yi *et al.*, 2014[a]). In addition to fat, the giving of bile fat (taurocholic acid) can increase carotene absorption and accumulation efficiency which gives significant effect on pigmentation (Olsen *et al.*, 2005; Yang *et al.*, 2012). Carotene absorption in intestine involves some steps which are feed matrix crushing, feed distribution in the form of fat emulsion then solubilization of bile fat mixture is performed formerly before it is transported to *enterocyte brush border* in which the absorption occurs (Furr and Clarck, 1997).

The giving of carotene materials adding on artificial feeds obviously needs to be given continuously. The termination of carotene materials adding on leopard grouper fish seeds for 3 months cause the decrease of carotenoids contain in fish body by 35.92% which is seen visually in which color performance on body part into brownish yellow while on anal and dorsal fins are dominated by yellow up to orange colors. While on caudal fin the decrease of pigmentation change has not appeared. This indicates that red pigmentation reduction is started on body area then anal and dorsal fins. Meanwhile, on caudal fin part, the pigmentation is more stable. This is

obviously caused by pigment deposition on chromatophore, which is also reported on yellow croaker fish (Yi *et al.*, 2014[b]), and on Australian snapper fish (Doolan *et al.*, 2008). However, the explanation on pigment deposition is still not able to be explained.

Based on confirmation of protein band on skin organ of leopard grouper fish seeds of D60 and D150 of all treatment show the number of the same allele variation which is 26 alleles. In the result of skin organ protein band confirmation of leopard grouper fish seeds of D60 it is seen that each treatment expresses specific protein band and in control treatment also shows 2 protein bands lost. This indicates that carotene giving from different sources express different specific protein. Obviously these are related to the type and contain containing in carotene materials source (Johnson and Lewis, 1979; Del Campo and García-González, 2007). Protein is expression of gen, phenotype character as interaction result between genotype and environment factors (Brock *et al.*, 1999). It is assumed that fish will express specific protein as response toward outside effect in this case is carotene. To find out the existence of protein triggered by carotene absorbed, protein analysis can be conducted. Protein profile analysis can be conducted by SDS-PAGE method which is protein separating method based on molecule weight difference (Bollagand Edelstein, 1991). Base on this, it can be said that red color pigmentation on leopard grouper fish of D60 is triggered by carotene materials giving especially phaffia and *haematococcus* kinds.

Meanwhile, on leopard grouper fish seeds of D150, on seeds which are still given phaffia and *haematococcus* carotene materials intake there is no specific protein band, but on seeds with carotene materials intake termination show protein band lost on molecule weight of 82.1 kDa and show 4 new protein bands (151.3 kDa, 81.4 kDa, 32.4 kDa, 24kDa). By seeing all protein band confirmation on seeds of D60 and D150, it is seen that protein band exist with molecule weight of 84.2 kDa (D60) and 82.1 kDa (D150) which appear of phaffia and *haematococcus* treatments which give the best red color performance. This profile is assumed to be related to protein expression related to astaxanthin which is deposition on skin tissues, because on control seeds of D60 and seeds of D150 with carotene materials intake termination experience protein lost in the molecular weight. It is known that carotenoids kind on integument system which is the most excessive on red marine fish is astaxanthin kind and the second is tunaxanthin (Tanaka *et al.*, 1976). Astaxanthin is carotenoid type which is responsible to red color performance (Storebakken and No, 1992) while tunaxanthin is carotenoid type which is isolated from blue fin tuna (Tanaka *et al.*, 1976). For another carotenoid type like canthaxanthin and 3-Hydroxy-canthaxanthin can be directly deposition into flesh without needing to be changed into astaxanthin(Tanaka *et al.*, 1976) and the most efficient astaxanthin form absorbed by fish is astaxanthin type (Choubert and Storebakken, 1989; Torrissen, 1989).

Conclusion

The giving of carotene materials of *Haematococcus pluvialis* gives the best red color performance with total value of carotene of 34.37% higher than *Phaffia rhodozyma* kind. Carotenoid should be remained given continuously as trigger to maintain red color performance on Leopard grouper grouper fish seeds. There is protein band in the range of 82.1 – 84.2 kDa which is assumed as expression of astaxanthin protein.

Suggestions

Efficiency in carotenoid absorption of *Haematococcus pluvialis* kind is the lowest. So the test using fat and bile fat needs to be conducted to increase carotenoids digestion and absorption in fish digestion system in order that carotene accumulation can increase and red color pigmentation on leopard grouper fish of cultivation result can balance natural leopard grouper fish.

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