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Effect of ascidian (*Halocynthia roretzi*, Drasche 1884) tunics carotenoids on enhancing growth and muscle coloring of sea-reared rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792)

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ABSTRACT

A 120 days trial was conducted to investigate the effect of sea squirt (*Halocynthia roretzi*, Drasche 1884) tunic's carotenoid to sea-reared rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) growth and muscle color. Sea-rearing was done at Tongyeong sea area, Korea. Three dietary treatments, namely control (C), CT, and AT, were administered to 6 groups of fish (n = 490-520). C was given basal diet which has contained 40 mg kg^{-1} of astaxanthin. A further inclusion of 10 mg kg^{-1} canthaxanthin was added to diet of CT, while AT's feed was supplemented with 10 mg kg^{-1} *H. roretzi* tunics carotenoids extract. The result revealed that AT has the highest final weight (1119.2 ± 82.4 g) compare to those of C (881.0 ± 121.2 g) and CT (1068.2 ± 4.3 g). The specific growth rate (SGR) of AT (1.0 ± 0.07%/day) was significantly higher than C (0.7 ± 0.22%/day) and CT (0.7 ± 0.25%/day) while the feed conversion ratio (FCR) were 1.5 ± 0.6, 1.4 ± 0.6, and 1.2 ± 0.1 for C, CT and AT respectively. The hepatosomatic index (HSI) and Viscerosomatic index (VSI) of all groups showed no significant difference ($p > 0.05$). The muscle color was also positively affected by the treatments, CT and AT were significantly different from C ($p < 005$). The initial muscle color score was 1.7 ± 0.0 and the final scores were 3.4 ± 0.2, 5.6 ± 0.1, and 5.7 ± 0.0 for C, CT, and AT respectively. Moreover, muscle carotenoids content of AT (8.5 ± 0.2 mg kg^{-1}) was significantly higher ($p < 0.05$) than those of CT (6.9 ± 0.3 mg kg^{-1}) and C (6.1 ± 0.2 mg kg^{-1}). Astaxanthin evidently is the most prominent carotenoid present in the muscle from all groups.

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) is noticed to have red color muscle and known to accumulate astaxanthin in its muscle (García-Chavarría and Lara-Flores, 2013). The degree of muscle pigmentation is a key attribute of this fish considering consumer acceptability (Jobling et al., 1998) and in which, its price depends upon (Metusalach and Brown, 1997). The typical redness of the muscle is due to the presence of carotenoids that the fish cannot synthesize in itself and must be obtained from its diet (Berjekeng et al., 1990). The muscle color of farmed salmonid is enhanced by adding two major carotenoids, namely canthaxanthin and astaxanthin into its diet (Choubert and Storebakken, 1996; Tolasa et al., 2005; Torrissen et al., 1989). Astaxanthin is one of the major carotenoid pigments present in aquatic animals (Christiansen & Torrissen 1997; Guerin et al., 2003) and has important biological functions, which include: prevention of the oxidation of unsaturated fatty acids; protection against the negative effects of ultraviolet light; action as pro-vitamin A; control of growth and reproductive behavior; and enhancement of the immune system (Bell et al., 2000; Lorenz and Cysewski 2000; Torrissen et al., 1989). The study by Nakano et al. (1995) on the effect of astaxanthin from *Phaffia rhodozyma* and synthetic astaxanthin to the biochemical characteristics of rainbow trout's liver and blood showed that carotenoids are also involved in certain physiological functions such as lower hepatic transaminase activities, protection of serum lipoprotein from auto-oxidation, and moreover carotenoids served as antioxidant.

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The positive effect of carotenoids on rainbow trout has been well documented as well as comparative pigmentation efficacy between astaxanthin and canthaxanthin (Choubert and Storebakken, 1996; Storebakken and No, 1992; Torrissen, 1986; Torrissen et al., 1989). Choubert et al. (2006) reported that fish fed with synthetic astaxanthin has higher retention of it in its muscle than fish fed with algae. Red algae, *Gracilaria vermiculophyllą* was also used as supplement in rainbow trout feed (Araújo et al., 2015). Both researchers agreed that carotenoids, either synthetic or algae original, have positive effect on rainbow trout growth and muscle coloring.

Wild fish derive carotenoids through their prey; while for intensive rearing system, it must be added to the feed (Halver and Hardy, 2002; Johnson and An, 1991). This process is considered costly for aquaculture industries (Gourveia et al., 1997; Hardy et al., 1990). To augment this problem, many studies on microalgae as the main sources of natural carotenoids were performed and are being adopted by feed industries (Del Campo et al., 2007; Yaakob et al., 2014).

To be able to utilize other sources of carotenoids, we investigated the effect of carotenoids extract from tunics of *Halocynthia roretzi* as alternative natural pigment source for sea-farmed rainbow trout. Sea squirt (*Halocynthia roretzi*, Drasche 1884) is an ascidian that is popular as seafood in Korea and Japan. It can be found in Southern and Eastern Sea of Korea and Northern sea of Japan (Rho et al., 1996). Aquaculture production of sea squirts in Korea reached 7038 t in 2014 and projected to reach 9977 t in 2015 (Korea Maritime Institute, 2015). The tunics of *H. roretzi,* the inedible part, are underutilized. Previous studies (Choi et al., 1994; Choi et al., 1996; Hong et al., 2001) showed it has high concentration of carotenoids and other valuable compounds. Choi et al. (1994) documented that *H. roretzi* tunics contain 13 types of carotenoids; 6 of which are the most abundant, namely: alloxanthin, canthaxanthin, halochintiaxanthin, diatoxanthin, diadinochrome, and mytiloxanthinone, as well as minor amounts of lutein, mytiloxanthin, astaxanthin, and β-carotene.

Lab scale study on *H. roretzi* tunic's carotenoids effect on rainbow trout muscle color has been conducted by Lee et al. (1994). The result showed that feeding 40–80 mg kg^{-1} astaxanthin equivalent of *H. roretzi* tunic's carotenoid extract for 8 weeks had similar coloration effect on rainbow trout muscle compare to those fed with astaxanthin (Carophyll Pink™) at same concentration. Based on the above mentioned study, the application of *H. roretzi* tunic's carotenoid to the bigger scale rainbow trout aquaculture would be investigated.

2. Materials and methods

2.1. Feed composition and fish culture

H. roretzi tunics were chopped (±2 cm^2) and soaked in acetone (10 × v/w) overnight. The acetone was filtered and evaporated using vacuum evaporator (Industry vacuum evaporator SP20, Hahn Shin Corp. Korea). The extract was then evaporated until dryness. Carotenoids extract was stored in an amber bottle, sealed under nitrogen gas and kept at −20 °C until utilization. The *H. roretzi* tunics contained 302.6 ± 1.3 mg kg^{-1} carotenoids, 19.0 ± 2.6% of lipid. Its fatty acids content was demonstrated in Table 1.

Aller Silver EX™ was used as basal diet obtained from Aller Aqua (DK-6070 Christiansfeld, Denmark). It contains 23.9 ± 0.1% of lipid, 48.1 ± 0.1% of Protein, 7 ± 0.3% of moisture, 8.5 ± 0.1% of Ash and 39.5 ± 0.8 mg kg^{-1} of Astaxanthin. Three feed treatments were prepared by addition of different concentrations and sources of carotenoids to the basal feed. Ten mg kg^{-1} of synthetic canthaxanthin (Carophyll Red® 10%) and 10 mg kg^{-1} carotenoid extract from *H. roretzi* tunics was added to CT and AT groups diet, respectively, while there was no further addition of carotenoid for C group.

Table 1
Fatty acid content of *H. roretzi* tunics carotenoid extract.

Fatty Acid	Concentration (%)	Fatty Acid	Concentration (%)
12:0	0.5 ± 0.1	16:2n6	0.6 ± 0.4
14:0	4.7 ± 0.3	18:2n6	2.2 ± 0.3
16:0	17.6 ± 0.4	18:3n3	1.5 ± 0.2
18:0	6.8 ± 0.5	18:3n1	0.2 ± 0.0
19:0	0.2 ± 0.0	18:4n3	1.0 ± 0.1
20:0	0.4 ± 0.1	20:3n6	0.1 ± 0.0
22:0	0.52 ± 0.1	20:3n4	0.4 ± 0.3
∑Saturates	37.9	20:3n3	1.0 ± 0.3
		20:4n6	4.5 ± 0.2
16:1n7	8.2 ± 0.7	20:5n3	7.9 ± 0.4
18:1n9	5.6 ± 0.1	22:5n6	1.6 ± 0.3
18:1n7	6.8 ± 0.1	22:6n3	5.8 ± 0.2
20:1n7	1.8 ± 0.3	∑Polyenes	34.3
22:1n13	1.1 ± 0.1		
22:1n9	1.0 ± 0.2	∑n-3	20.7
22:1n7	1.0 ± 0.1	∑n-6	10.2
∑Monoenes	27.8	Ratio n-3/n-6	2.0

Values are means ± sd (n = 6).

Rainbow trout (280.0 ± 21.5 g) was obtained from fresh water trout nursery at Sancheon, Korea. The fish were transported live to open sea farm at Tongyeong, Korea. On arrival at seaport, the fresh water was gradually changed into seawater in less than 3 h. Three thousand of fish were distributed into 6 sea pens (12 m × 12 m × 7 m) equally. These were acclimatized for 3 weeks. During acclimatization, they were given basal diet. Feed were given twice a day at maximum portion of 3% BW to ensure fish satiety. By the end of experiment, each cage was given 2.4 ± 0.2 tons of feed.

Two pens each were designated for C, CT, and AT. Feeding trial was carried out for 120 days from December 2013-May 2014 with water temperature recoded as: 9.8 ± 1.0 °C, 9.5 ± 0.8 °C, 11.1 ± 1.1 °C, 13.1 ± 2.1 °C, 16.8 ± 3.5 °C, 18.2 ± 1.2 °C for each month respectively.

2.2. Growth parameters

Six fish from each pen were captured at the end of acclimatization to obtain initial data (day 0). Another six fish from each pen were captured randomly every 30 days. These were then subjected to measurement of its standard and total length as well as total body, muscle, viscera, and liver weights. The growth performance was assessed using the following equations: Specific Growth Rate (SGR) = [(ln (final weight)-ln (initial weight))/days of treatment] × 100, and Feed Conversion Ratio (FCR) = Dry weight of given feed/body weight gain. The condition of the fish were evaluated through several indices, namely hepatosomatic index (HIS) = 100 × (liver weight/total body weight); viscerosomatic index (VSI) = 100 × (viscera weight/total body weight); Muscle Index (MI) = 100 × (muscle weight/total body weight), in which the muscle weight was the weight of skinless and bone free filet (Anderson and Neumann, 1996). Biometric analysis and muscle color observations were done posthaste on fresh samples. All samples were kept at −40 °C until further experiments.

2.3. Chemical analysis

The muscle of rainbow trout was subjected to general proximate composition. Moisture, crude protein and ash were determined by the methods described by the Association of Official Analytical Chemists (AOAC, 1995). Lipids were extracted with chloroform/methanol (2:1 v/v) in accordance with the procedure of Bligh

Table 2
Means (±SE) of weights and growth indices of sea-reared rainbow trout (*Oncorhynchus mykiss*) fed with different level and sources of dietary carotenoids.

	Days of treatment	Group		
		C	CT	AT
Weight (g)	0	$391.2 \pm 2.7^{a,w}$	$390.5 \pm 0.0^{a,w}$	$338.0 \pm 0.0^{b,w}$
	30	$416.4 \pm 7.9^{a,w}$	$507.8 \pm 45.2^{a,w}$	$512.5 \pm 72.2^{a,w}$
	60	$548.9 \pm 61.9^{a,w}$	$988.3 \pm 234.6^{b,x}$	$1074.5 \pm 12.5^{b,x}$
	90	$820.2 \pm 23.9^{a,x}$	$1067.9 \pm 36.6^{a,x}$	$1096.2 \pm 113.6^{b,x}$
	120	$881.0 \pm 121.2^{a,x}$	$1068.2 \pm 4.3^{ab,x}$	$1119.2 \pm 82.4^{b,x}$
Indices:				
SGR (%/day)[1]		0.7 ± 0.22^{a}	0.7 ± 0.25^{a}	1.0 ± 0.07^{b}
FCR[2]		1.5 ± 0.6^{b}	1.4 ± 0.6^{b}	1.2 ± 0.1^{a}
VSI[3]		16.1 ± 0.0^{a}	16.2 ± 0.0^{a}	16.6 ± 0.0^{a}
HSI[4]		1.5 ± 0.1^{a}	1.6 ± 0.1^{a}	1.5 ± 0.1^{a}
MI[5]		83.5 ± 4.4^{a}	87.8 ± 1.5^{ab}	90.6 ± 1.3^{b}

Values are means ± sd (n = 12).
Means in the same row with different letters (a,b) are significantly different from each other ($p < 0.05$).
Weight's means in the same column with different letters (w,x) are significantly different from each other ($p < 0.05$).
[1] Specific growth ratio = [(ln (final weight)-ln (initial weight))/days of treatment] × 100.
[2] Feed conversion ratio = Dry weight of given feed/body weight gain.
[3] Viscerosomatic index (VSI) = 100 × (viscera weight/total body weight).
[4] Hepatosomatic index (HSI) = 100 × (hepatosomatic weight/total body weight).
[5] Muscle Index (MI) = 100 × (muscle weight/total body weight).

Table 3
Proximate composition of muscle of sea-reared rainbow trout (*Oncorhynchus mykiss*) fed with different level and sources of dietary carotenoids.

Composition (g kg^{-1})	Group	Treatment time (Days)				
		0	30	60	90	120
Ash	C	58.2 ± 1.0^{a}	52.2 ± 6.0^{a}	53.8 ± 2.5^{a}	58.4 ± 0.9^{a}	$50.6 \pm 0.7^{a,x}$
	CT	52.4 ± 0.6^{bc}	48.4 ± 2.4^{ab}	46.1 ± 7.4^{ab}	60.6 ± 0.8^{c}	$41.2 \pm 0.8^{a,y}$
	AT	49.2 ± 0.3^{a}	45.7 ± 5.6^{ab}	47.7 ± 3.4^{ab}	48.8 ± 0.2^{ab}	$41.2 \pm 0.4^{b,y}$
Moisture	C	723.0 ± 5.2^{d}	705.5 ± 5.8^{c}	669.9 ± 1.0^{c}	663.9 ± 2.3^{b}	$640.8 \pm 12.8^{a,x}$
	CT	718.3 ± 5.7^{c}	712.9 ± 4.8^{c}	668.8 ± 10.7^{b}	635.5 ± 4.3^{a}	$628.9 \pm 8.7^{a,xy}$
	AT	722.7 ± 6.8^{d}	690.3 ± 15.2^{c}	644.6 ± 9.0^{b}	640.5 ± 3.4^{ab}	$619.8 \pm 5.9^{a,y}$
Lipid	C	10.4 ± 0.5^{a}	21.4 ± 1.3^{b}	22.6 ± 0.2^{bc}	23.5 ± 1.4^{bc}	24.1 ± 0.1^{cx}
	CT	13.9 ± 0.1^{a}	15.0 ± 1.0^{a}	19.0 ± 0.3^{b}	19.7 ± 0.1^{b}	$22.6 \pm 2.3^{c,xy}$
	AT	12.5 ± 0.0^{a}	14.1 ± 1.5^{a}	21.8 ± 0.2^{b}	26.0 ± 0.1^{c}	$25.8 \pm 0.3^{c,y}$
Protein	C	125.9 ± 2.5^{a}	149.2 ± 5.0^{b}	170.9 ± 7.6^{b}	181.1 ± 6.4^{c}	$230.7 \pm 4.7^{d,x}$
	CT	125.8 ± 0.1^{a}	154.0 ± 4.2^{b}	187.2 ± 5.6^{c}	225.1 ± 4.3^{d}	$234.0 \pm 5.0^{d,xy}$
	AT	125.9 ± 2.5^{a}	188.5 ± 6.8^{b}	206.9 ± 10.6^{c}	221.6 ± 1.8^{cd}	$238.8 \pm 3.4^{d,y}$

Values are means ± sd (n = 12). Means in the same row with different letters are significantly different from each other ($p < 0.05$). Means at 120 days of treatment column with different letters (x,y) are significantly different from each other ($p < 0.05$).

and Dyer (1958). The extracts were evaporated on a rotary evaporator and the total lipid content was determined gravimetrically. Carotenoid content of the muscle was analyzed in accordance with the method of Rodriguez-Amaya (1999).

2.4. Muscle color analysis

Muscle color was observed visually by comparing the free skin and bone muscle from ventro-cranial dorsal fin area (1.5 cm thick, 2 cm in diameter) to Salmonids Roche™ Color Card. The observation was carried out under natural light and evaluated by three independent observers. Instrumental color L* (lightness), a* (redness) and b*(yellowness) were measured using a Minolta Chroma Meter CR-300 (Illuminant D65, Minolta Co., Ltd., Osaka, Japan), observer angle 2° and calibrated against white tile. Color measurements were taken after exposure of the muscle surface into the air for 10 min. For every fish, measurements were performed twice on two adjacent muscle samples taken from the ventro-cranial portion of the dorsal fin. Chroma (C*, CIE Lab color space), numerically quantified as $[C^* = (a^{*2} + b^{*2})^{1/2}]$ (Lindahl et al., 2001; McNiven et al., 2012).

2.5. Carotenoid HPLC analysis

Sixty milligram of muscle from ventro-cranial dorsal fin area was crushed and mixed with 10 mL cold acetone. The mixture was then centrifuged at 4 °C, 1500*g* for 10 min. The supernatant was transferred into clean glass test tube and vortexed after addition of 10 mL hexane and 5 mL10% NaCl. Once the aqueous phase had settled, the water free upper layer was removed and evaporated to dryness and then redissolved in 2 mL of mobile phase. The solution was mixed thoroughly, filtered with 0.2 μm filter into an amber vial, and kept at −20 °C prior to chromatography. The high performance liquid chromatography (HPLC) analysis was performed within 3 h after carotenoid extraction.

Muscle carotenoid extracts were then analyzed using the HPLC (Shimadzu, Japan) fitted with YMC (Tokyo, Japan) C30 column (4.6 mm × 250 mm, 5 μm) and column guard Phenomenex® C18. Mobile phases were methanol-water (92:8) as solvent A and 100% *tert*-butyl methyl ether as solvent B. The gradient condition was 80% A at the beginning, decreased to 20% A in 48 min, then returned to 80% A in 49 min and maintained until 54 min. Flow rate was 0.8 mL/min and detection was at 450 nm (Inbaraj et al., 2006; Kao

Table 4
Color measurements of sea-reared rainbow trout (*Oncorhynchus mykiss*) muscle with different level and sources of dietary carotenoids treatment.

		Muscle Color[1]	L*[2]	a*[2]	b*[2]	C*[3]
C	0	1.6 ± 0.1^{a}	64.1 ± 0.2^{h}	5.7 ± 0.2^{ab}	3.6 ± 0.0^{d}	20.5 ± 0.2^{a}
	30	2.7 ± 0.6^{b}	61.8 ± 0.3^{g}	6.2 ± 0.1^{bcd}	3.7 ± 0.0^{f}	22.3 ± 0.5^{ab}
	60	2.0 ± 0.0^{b}	60.9 ± 0.2^{g}	6.6 ± 0.3^{def}	4.6 ± 0.0^{n}	25.0 ± 1.4^{bc}
	90	2.8 ± 0.3^{bc}	57.8 ± 0.5^{de}	6.7 ± 0.1^{efg}	4.6 ± 0.0^{m}	27.4 ± 0.6^{cd}
	120	3.4 ± 0.2^{c}	54.2 ± 0.3^{b}	6.9 ± 0.1^{fgh}	4.5 ± 0.0^{l}	27.8 ± 1.1^{cd}
CT	0	1.6 ± 0.1^{a}	63.9 ± 0.2^{h}	5.6 ± 0.1^{a}	3.6 ± 0.0^{e}	20.4 ± 0.2^{a}
	30	3.0 ± 0.2^{b}	59.6 ± 0.6^{f}	6.2 ± 0.2^{cde}	3.3 ± 0.0^{b}	21.0 ± 1.0^{a}
	60	3.7 ± 0.6^{cd}	58.5 ± 0.6^{ef}	7.1 ± 0.2^{gh}	4.0 ± 0.0^{g}	26.5 ± 0.9^{cd}
	90	4.4 ± 0.2^{d}	55.9 ± 0.4^{c}	7.3 ± 0.2^{hi}	4.5 ± 0.0^{l}	29.4 ± 1.1^{d}
	120	5.6 ± 0.1^{e}	54.1 ± 0.2^{b}	7.7 ± 0.1^{ij}	4.5 ± 0.0^{l}	28.9 ± 0.5^{d}
AT	0	1.5 ± 0.1^{a}	64.1 ± 0.3^{h}	5.7 ± 0.2^{abc}	3.6 ± 0.0^{a}	20.3 ± 0.2^{a}
	30	3.0 ± 0.3^{b}	58.1 ± 0.5^{e}	7.3 ± 0.3^{hi}	3.2 ± 0.0^{c}	26.8 ± 1.5^{cd}
	60	3.7 ± 0.5^{c}	56.7 ± 0.4^{cd}	7.8 ± 0.1^{ij}	4.0 ± 0.0^{i}	32.8 ± 2.0^{e}
	90	4.6 ± 0.2^{d}	54.4 ± 0.6^{b}	7.9 ± 0.1^{j}	4.0 ± 0.0^{h}	35.6 ± 1.3^{ef}
	120	5.7 ± 0.0^{e}	52.0 ± 0.2^{a}	8.1 ± 0.2^{j}	4.1 ± 0.0^{k}	36.7 ± 0.4^{f}

Values are means ± sd (n = 12). Means in the same column with different letters are significantly different from each other ($p < 0.05$).
[1] Muscle Color was observed by comparing muscle sample to Salmonids Roche™ Color Card.
[2] L* (lightness), a* (redness) and b*(yellowness) were measured using a Minolta Chroma Meter *CR*-300 (Minolta Co., Ltd., Osaka, Japan).
[3] Chroma (C*) = $(a^{*2} + b^{*2})^{1/2}$.

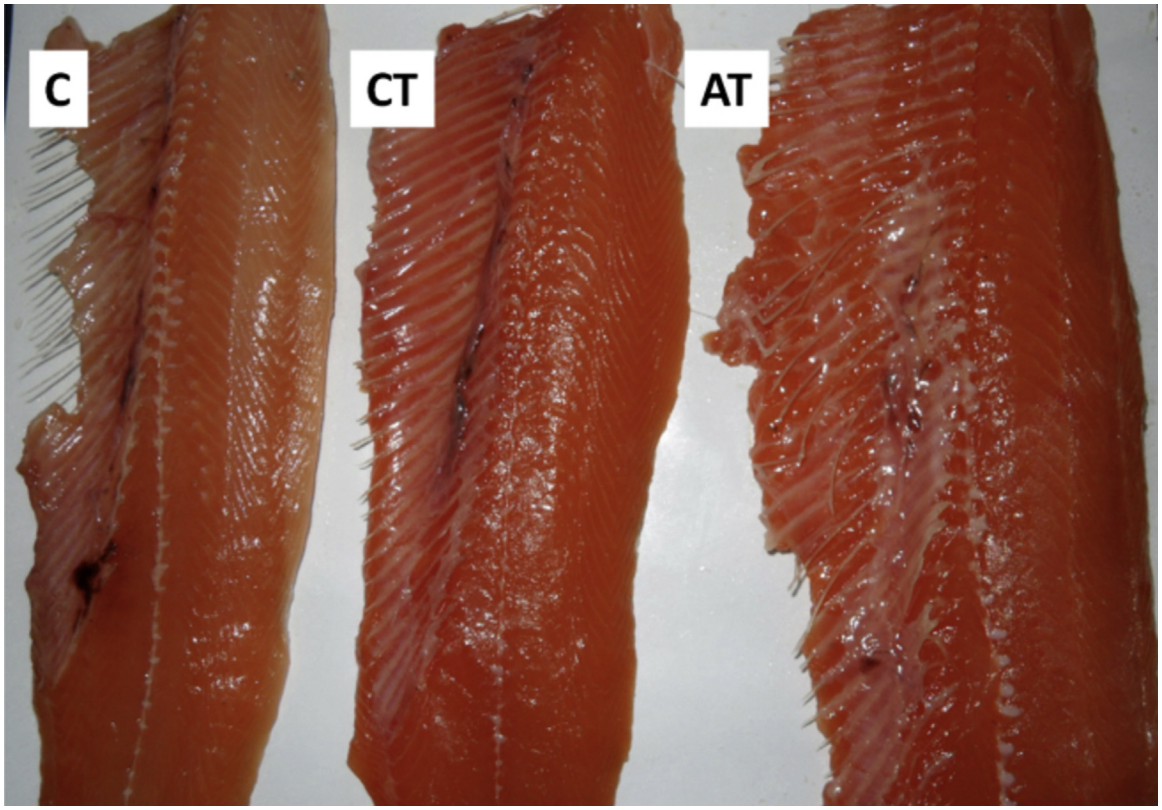

Fig. 1. Fillet of sea-reared rainbow trout treated with 40 mg kg^{-1} of astaxanthin (C), 40 mg kg^{-1} astaxanthin+ 10 mg kg^{-1} of canthaxanthin (CT), and 40 mg kg^{-1} Astaxanthin + 10 mg kg^{-1} ascidian tunic carotenoid extract (AT) for 120 days.

et al., 2012). A series of dilution of standards (Carotene (Sigma Aldrich Co., USA), Astaxanthin from *Haematococcus pluvailis* (Sigma Aldrich Co., USA), Canthaxanthin (Sigma Aldrich Co., USA), and Lutein (HKBiotech, Korea)) were used as references. In addition, the carotenoid extract of the tunics was used for comparison of retention time and absorption spectra. Unknown peaks were identified based on absorption spectra characteristics as described in previous studies (Choi et al., 1994; Inbaraj et al., 2006).

2.6. Data analysis

The data obtained during the course of this experiment was analyzed statistically using Minitab® 17.1.0. (Pennsylvania, USA). Each set of data was analyzed with one-way analysis of variance (ANOVA) and differences were considered significant when $p < 0.05$. Least Significant Difference (LSD) test and Tukey's range test were performed as post-ANOVA comparison of means in order to determine any differences.

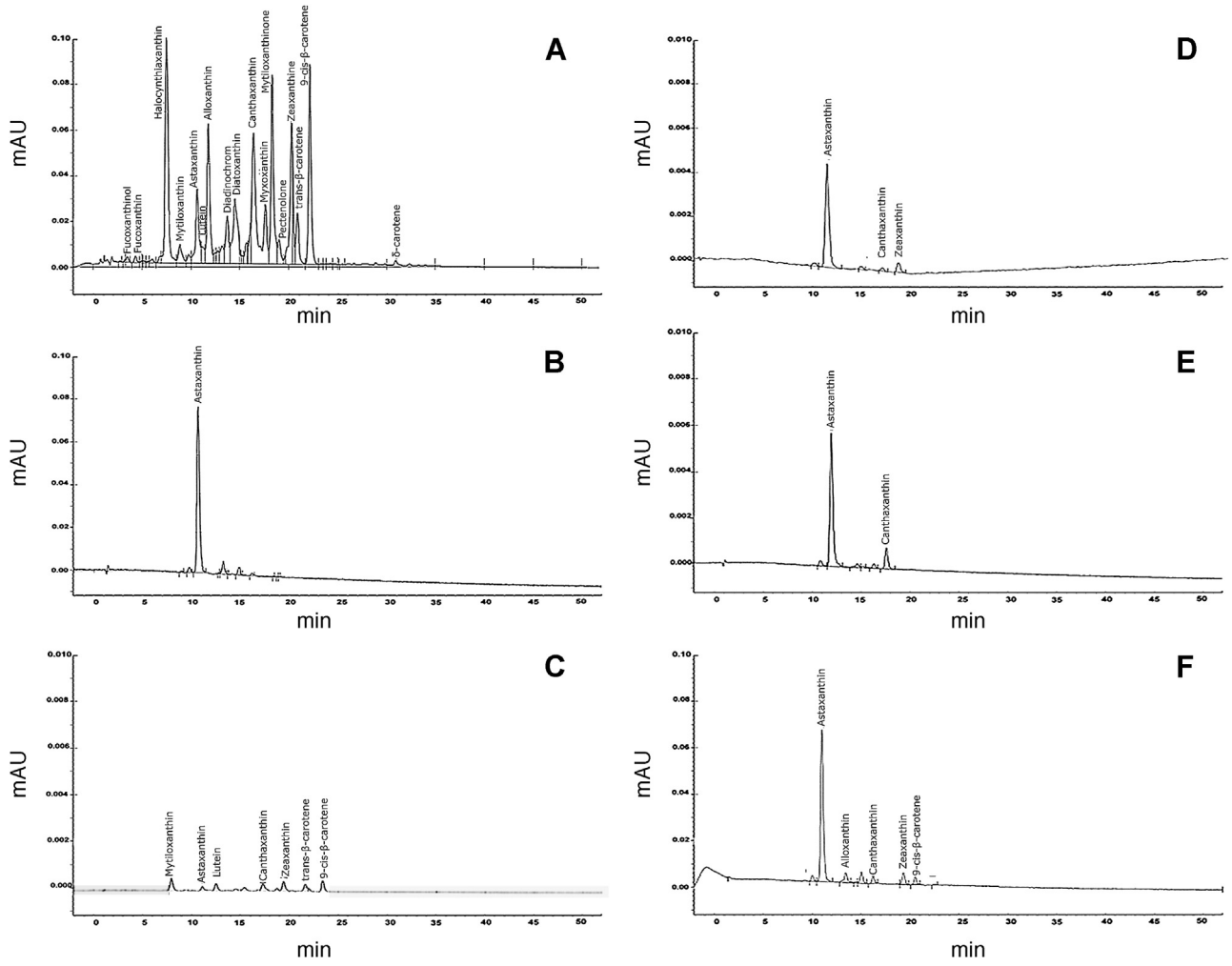

Fig. 2. Representative HPLC chromatogram of carotenoids from ascidian tunics extract (A), basal feed Aller silver EX™ (B), rainbow trout muscle 0 day (C), and after 120 days of treatment for group C (D), CT (E), and AT (F). Samples were applied to an YMC (Tokyo, Japan) C30 column (4.6 mm × 250 mm, 5 μm) with column guard (Phenomenex® C18). Mobile phases were methanol-water (92:8) as solvent A and 100% *tert*-butyl methyl ether as solvent B. The gradient condition was 80% of A at the beginning, decreased to 20% of A in 48 min, then returned to 80% of A in 49 min and maintained until 54 min. Flow rate was 0.8 mL/min and detection at 450 nm. The peaks were labelled according to the compound it represents.

3. Results

3.1. Growth and feed utilization

Table 2 shows AT group had highest final weight (1119.2 ± 82.4 g) compared to CT (1068.2 ± 4.3 g) and C (881.0 ± 121.2 g). AT was significantly higher than C ($p < 0.05$). Addition of 10 mg kg^{-1} *H. roretzi* tunic carotenoids extract (AT) had increased SGR (1.0 ± 0.07) significantly compared to CT (0.7 ± 0.25) and C (0.7 ± 0.22). FCR values were 1.5 ± 0.6, 1.4 ± 0.6, and 1.2 ± 0.1 for C, CT and AT, respectively. MI showed similar pattern as SGR and FCR. No significant differences ($p > 0.05$) were found for VSI and HSI among groups (Table 2).

3.2. Proximate compositions of rainbow trout muscle

The proximate composition is presented in Table 3. Protein and lipid content of fish muscle were inclined over time of treatment. The highest protein content in all treatments groups was achieved after 120 days of treatment, which were 230.7 ± 4.7, 234.0 ± 5.0, and 238.8 ± 3.4 g kg^{-1} for C, CT, and AT, respectively. These were almost double from their initial level at 0 day of treatment. This inclination was also found in lipid content of the muscle. On the other hand, the moisture contents of all groups declined during the course of the treatment.

3.3. Muscle coloring and carotenoids content

Color assessment of muscle is presented in Table 4 and Fig. 1. In the course of experiment, all groups were observed to have muscle color enhancement as reflective on the scales of muscle coloration after 120 days of treatments. These were 3.4 ± 0.2, 5.6 ± 0.1, and 5.7 ± 0.0 for C, CT, and AT, respectively. There was no significant difference between CT and AT ($p > 0.05$), but both are significantly different from C ($p < 0.05$). The degree of lightness (L^*) were 54.2 ± 0.3, 54.1 ± 0.2, and 52.0 ± 0.2, while the redness (a^*) were 6.9 ± 0.1, 7.7 ± 0.1, and 8.1 ± 0.2 for C, CT, and AT, respectively. Yellowness (b^*) of the C, CT, and AT muscle were 4.5 ± 0.0, 4.1 ± 0.0, and 4.1 ± 0.0 respectively. Based on the L^*, a^* and b^* measurements, CT and AT were observed as darker, redder and less yellow than C. For the calculation of chroma (C^*), it was seen that AT had the highest value (36.3 ± 0.4) compared to CT (28.9 ± 0.5) and C (27.8 ± 1.1).

The carotenoids content of sea reared rainbow trout muscle is shown in Table 5 and Fig. 2. Group C had the lowest total carotenoids content (6.1 ± 0.2 mg kg^{-1}) and AT had the high-

Table 5
Carotenoid content of sea-farmed rainbow trout (*Oncorhynchus mykiss*) muscle treated with different level and sources of dietary carotenoids.

Carotenoid (mg kg^{-1})	C					CT					AT				
	0	30	60	90	120	0	30	60	90	120	0	30	60	90	120
Halocynthiaxanthin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.5 ± 0.0	0.7 ± 0.0	TR	TR
Mytiloxanthin	0.3 ± 0.0	0.4 ± 0.0	TR	0.1 ± 0.0	TR	0.3 ± 0.0	TR	TR	TR	TR	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	TR	TR
Astaxanthin	0.1 ± 0.0	0.5 ± 0.0	2.9 ± 0.2	3.4 ± 0.2	5.4 ± 0.1	0.1 ± 0.0	3.9 ± 0.1	5.3 ± 0.1	6.3 ± 0.1	6.3 ± 0.1	0.1 ± 0.0	2.3 ± 0.1	2.6 ± 0.1	6.9 ± 0.1	7.1 ± 0.1
Lutein	0.2 ± 0.0	0.2 ± 0.0	TR	0.1 ± 0.0	TR	0.2 ± 0.0	0.3 ± 0.0	TR	TR	TR	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	TR
Alloxanthin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0
Cantaxanthin	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
Zeaxanthin	0.3 ± 0.0	0.3 ± 0.0	TR	TR	TR	0.3 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	TR	TR	0.3 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	TR	0.5 ± 0.0
Trans-β-carotene	0.2 ± 0.0	0.3 ± 0.0	TR	0.1 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	TR	TR	TR	0.2 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
9-cis-β-carotene	0.3 ± 0.0	0.4 ± 0.0	TR	0.1 ± 0.0	TR	0.3 ± 0.0	0.1 ± 0.0	TR	TR	TR	0.3 ± 0.0	0.1 ± 0.0	0.7 ± 0.0	TR	TR
Total Carotenoid	1.6 ± 0.4^{a}	2.4 ± 0.4^{ab}	3.1 ± 0.2^{b}	4.1 ± 0.3^{c}	6.1 ± 0.2^{d}	1.5 ± 0.2^{a}	4.3 ± 0.7^{c}	6.0 ± 0.3^{d}	6.3 ± 0.3^{d}	6.9 ± 0.3^{de}	1.6 ± 0.4^{a}	6.0 ± 0.3^{d}	6.6 ± 0.3^{d}	7.7 ± 0.2^{ef}	8.5 ± 0.2^{f}

Values are means ± sd (n = 6). Means in the same row with different letters are significantly different from each other ($p < 0.05$).

est (8.5 ± 0.2 mg kg^{-1}). Astaxanthin was found out as the major carotenoid among all groups. Group AT (Fig. 2F) had traces of halocynthiaxanthin and alloxanthin. These two carotenoids were recognized as particular carotenoids found in *H. roretzi* tunic carotenoid extract (Fig. 2A).

4. Discussion

Carotenoid is classified as micronutrient for fish. This is usually added as feed attractant (Hardy and Barrows, 2002). For red muscle fish, like rainbow trout, addition of carotenoids during culture will enhance muscle color (Simpson et al., 1981; Storebakken and No, 1992; Torrissen et al., 1989).

Lee et al. (1994) study showed that rainbow trout fed with 40–80 mg kg^{-1} astaxanthin equivalent of *H. roretzi* tunic's carotenoid for 8 weeks were scored the highest for customer preferences and its muscle color had no difference with those treated with astaxanthin (Carophyll pink^{TM}) at same concentration. Based on those results, lower concentration of carotenoids (50 mg kg^{-1}) was applied for the longer time of treatment (120 days) in this experiment. Furthermore, canthaxanthin (Carophyll red^{TM}) was chosen as one of the treatment since *H. roretzi* tunic's carotenoid contain high amount of it as described by Choi et al. (1994).

In this study, the feed base (Aller silver EX^{TM}) had contained 40 mg kg^{-1} of astaxanthin. The attractants were added to the feed to give a uniform taste for the fish and a better feed intake (Aller Co., 2012). Addition of 10 mg kg^{-1} carotenoids into this basal diet evidently increased the final weight of sea reared rainbow trout (Table 1). This event could be accounted to the nature of carotenoids as protection of lipid from auto-oxidation (Bell et al., 2000; Simpson et al., 1981). Lipid is known as one of the main energy sources for fish (Halver and Hardy, 2002). The addition of carotenoids to the feed will prevent excessive auto-oxidation thus making the lipid highly available for the fish to utilize; consequently more energy was available for fish metabolism and growth.

Carotenoids extract of *H. roretzi* tunics is also rich in lipid and other lipid soluble compounds. It consists of 17.6% of palmitic acid, 2.2% linolenic acid, 7.9% EPA, and 5.8% DHA (Table 1). The presence of carotenoids and these long chain omega-3 fatty acids from *H. roretzi* carotenoids extract contributed to the growth of sea-reared rainbow trout and increased its efficiency to convert feed into muscle as reflected in SGR, FCR, and MI value of AT (Table 2). This result was in alliance with study of Kurnia et al. (2015), whom reported positive effect of dietary carotenoids on growth and feed efficiency on rainbow trout. Furthermore, the importance of the addition of long chain omega-3 fatty acids to fish feed was affirmed by Watters et al. (2012). Fish require these fatty acids for optimal growth and development. Tailoring the nutritional composition of fish feed to meet the physiological needs of fish can improve fish development.

From the results, it can be seen, that the addition of *H. roretzi* tunics carotenoids extract for 120 days did not give rise to any deleterious effect on sea-reared rainbow trout. On the other hand, these result indicated that carotenoids extract of *H. roretzi* tunics enhanced the nutritional properties of the feed. This was in contrast with Bell et al. (1993) which reported that excessive dietary lipid can cause cardiovascular disorder on salmon especially under stress conditions.

From the consumer point of view, percentage of lipid and protein in the muscle, along with the volatile compounds will determine the taste of the fish (Kayim et al., 2010; Olafsdottir and Fleurence, 1998). The distinguished taste of fish muscle is preferred by consumer (Nakagawa, 2007), especially for high value fish like rainbow trout. In this experiment, groups fed with higher level carotenoids (CT and AT) had higher lipid and protein content, while the moisture was lower. This trend supposedly would accen-

tuate the special taste of rainbow trout muscle; hence it would be favorable for consumers.

All of the treatment groups showed increasing muscle color scales over time. At the end of the treatment, AT achieved the highest redness among the groups. The muscle color scale of C compare to CT and AT were 3.4 < 5.6 < 5.7, respectively (Table 4). Fig. 1 showed that after 120 days of treatment, CT and AT muscle appeared distinctively redder compare to C. These results were reasonable, since C was treated with the lowest concentration of carotenoids in the feed (40 mg kg^{-1}). Meanwhile, CT and AT muscle had comparable redness (Table 4, Fig. 1). Both groups were fed with 50 mg kg^{-1} carotenoids. In this experiment, rainbow trout muscle color parameters responded accordingly to the treatments. Albeit of the muscle color scale indicated that there was no significant difference ($p > 0.05$) between CT (5.6 ± 0.1) and AT (5.7 ± 0.0), carotenoids content (Table 5) and instrumental muscle color (Table 4) showed otherwise. As shown in Table 4, Chroma (C^*) was positively affected by diets. Lightness (L^*) decreased while a^* and b^* would increase over time of carotenoids feeding. This is in agreement with previous findings (Choubert and Luquet, 1982).

The majority of the studies on carotenoids concluded that astaxanthin was the most important carotenoid for salmonids and is better accumulated than canthaxanthin in rainbow trout (Baker, 2002). This study was in agreement with that trend, the HPLC analysis showed that astaxanthin was accumulated in muscle faster compare to other carotenoids (Table 5). In addition, the result showed that other carotenoids given in the feed could also effectively store on rainbow trout muscle and contributed to muscle color (Table 5, Fig. 2). The detection of particular carotenoids from *H. roretzi* on the rainbow trout muscle indicated that carotenoids from *H. roretzi*s tunics were easy to be absorbed and retained in muscle.

5. Conclusion

This study was conducted in southern sea area of Korea. Its water temperature is increasing quickly during spring to summer, thus is not suitable for cold water fish rearing and would hampers fish growth. The present result showed that supplementing 10 mg kg^{-1} carotenoids from *H. roretzi* tunics into basal feed that initially contained 40 mg kg^{-1} astaxanthin would have a positive effect to sea reared rainbow trout growth performance and muscle coloring. The result of this study could provide as a breakthrough in cold water fish farming at southern sea area of Korea. Also, this study will promote the utilization of *H. roretzi* tunics as alternative source of carotenoids for fish diet supplement.

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