Advances in Bioresearch
Adv. Biores., Vol 8 (2) March 2017: 109-114
©2017 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.8.2.109114

Advances in Bioresearch

ORIGINAL ARTICLE

The Effect of Diets with *Haematococcus pluvialis* Algae on the Flesh Color of *Oncorhynchus mykiss*

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ABSTRACT
This research aims to compare the effect of various amounts of meal of Haematococcus pluvialis algae on the flesh color of Rainbow trout (Oncorhynchus mykiss) with approximate weight of 140 g per fish. Fish of the five under study treatments were fed with meal of Haematococcus pluvialis algae in three replications and amounts of 0,25,50,75, 100 ppm astaxanthin. After 90 d feeding, the color change of fish muscle was investigated using visual evaluation and colorimeter techniques. The pigment concentration in fish muscles also was measured using UV-Vis spectrophotometer. Increasing of the amount of the meal of Haematococcus pluvialis algae and subsequently that of astaxanthin in the fish diet, scores of color of fish muscle, in the visual evaluation, also get increased. So that color scores of muscle of control fish, fish fed with diet lack of pigment were significantly lower than that of fish with other treatments ($p<0.01$). Fish of treatment 4, fed with diet containing 100ppm astaxanthin, had higher scores of muscle color compared with fish with other treatments ($p<0.01$).Colorimetric results showed decrease of lightness (L^*) and hue ($H(°)_{ab}$) and also increase of chroma (c^*), and red (a^*) and yellow (b^*) chromaticity. So muscle of treated control fish compared to that of fish of other treatment had the highest ($p<0.01$) color value and hue $H(°)_{ab}$ and the lowest ($p<0.01$) c^* and color values of a^* and b^*. Treatments 1, 2 and 3 displayed intermediate values of color muscle of fish. In addition to these values, the total values of carotenoid obtained for control treated fish muscle was significantly lower compared to that of fish of other treatments ($p<0.01$).Muscles of fish of treatment 4, compared to that of fish of other treatments, had more pigment concentration($p<0/01$). So, according to the present study we can conclude that data obtained from different methods of evaluation of color flesh of fish fed with Haematococcus pluvialis algae have provided reasonable values.
***Key words**: Haematococcus pluvialis, Flesh color,Oncorhynchus mykiss, Astaxanthin*

Received 21/11/2016 Revised 12/01/2017 Accepted 19/02/2017

How to cite this article:
M Amiri, H Emadi, M Emtyazjoo. The Effect of Diets with *Haematococcus pluvialis* Algae on the Flesh Color of *Oncorhynchus mykiss*. Adv. Biores., Vol 8 [2] March 2017: 109-114.

sINTRODUCTION

Aquaculture has some potential advantages over capture fisheries in terms of product quality because of its genetic selection of superior strains, controlling diet, culture environment, harvest methods and delivery product to market [1].In order to improvement of dietary values of product and customer satisfaction, the appearance and flavor of the cultured fish may change compared to that of wild caught counterpart [2]. Color is one of the most important quality traits of fish flesh [3, 4, and 11]. Consumers attribute the deep pink color of fish to the high quality[5].The special red to pink color of fish flesh is due to astaxanthin (3, 3′-dihydroxy-β, β-carotene-4, 4′-dione), a carotenoid with dietary origin that fish cannot produce it [6]. The meal of *Haematococcus pluvialis* algae (NatuRose™) provides a mixture of monoester, diester and free astaxanthin in a proportion similar to that of natural Krill [7].

One of first published reports studied the effect of completing the diet of Rainbow trout with the meal of *Haematococcus pluvialis* algae during 100 days of feeding test. Values of total mean of Carotenoids and astaxanthin were measured in the flesh and skin of Rainbow trout. This study showed that meal of *Haematococcus pluvialis* algae leads to the significant reservation of astaxanthin in the flesh and skin of Rainbow trout and significant increase of color of flesh [9].Another published test used the *Haematococcus* cells in 4 weeks of feeding test of Rainbow trout for comparison of color of muscle texture

with that of artificial Carotenoid. This study proved that astaxanthin and canthaxanthine which constitute 85 percent of total Carotenoids lead the significant pigment reservation in the muscle of Rainbow trout. These authors concluded that meal of *Haematococcus pluvialis* algae is a safe and effective source of pigment for Rainbow trout [9]. Different sources of pigment (natural or artificial) made color values in the fresh fillet without any significant difference. In a test conducted by Choubert [10] the effect of pigmentation of astaxanthin obtained from the green *Haematococcus pluvialis* microalgae and artificial astaxanthin in Rainbow trout was studied. During 6 weeks feeding, comparison the value of L* with primary sample L* showed significant decrease in fish fed with artificial astaxanthin compared to fish fed with *Haematococcus pluvialis* algae. In contrast, except hueH $(°)_{ab}$, which didn't show any difference in diets of fish, other color indices got increased. The chroma c*, a* and b* obtained for fish fed with artificial astaxanthin was higher than that of obtained for fish fed with algae. These results are consistent with previous data [10].For investigation about effects of complement of diets of Rainbow trout with microalgae rich in carotenoid, a dietary test was conducted by Sommer et al [8, 9].In this test, for the groups receiving supplements of pigment, the scores of flesh color and total amounts of carotenoid and astaxanthin increased in fish flesh.

This study performed for comparison of the effects of different values of meal of *Haematococcus pluvialis* algae on the flesh color of Rainbow trout during 90 days.

MATERIALS AND METHODS

Fish and nutritional management

This study was performed in Culture and Proliferation Center of Coldwater fish of Jajrood (Northeast of Tehran). Before feeding with test diets and during compatibility period, 450 numbers of Rainbrow trout (*Oncorhynchus mykiss*) fish were fed with DAN commercial diet lack of astaxanthin. When they gained weight of 140 per fish, were divided into five groups and kept in cement pools (240×85×66 cm) located in parallel rows, with water resource of well (temperature mean of 16/3±1/19; PH mean of 7/6; dissolved oxygen mean of 7/5 mg/L) and flow of 1-1/5l/s. the pools received a natural light period (10 July-10 October). Then fish were fed with 5 test diets in three repetitions (30 fish in each pool) for 90 d.Fish were fed twice a day (9 and 15 clock) in 3 percent of body weight in day.Fortnightly 60 numbers of fish of each bio- assayed treatments, and rate of daily diet based on gaining weight of fish and water temperature were computed.

Test diets

The five kinds of test diets used in this study were DAN foods (GFT-2, Chine Company, Iran) which were prepared with different values of the meal of *Haematococcus pluvialis* algae 1/5 percent (NatuRose™, Cyanotech Company, Kona, Hawaii). In order to this, a suitable concentration of starch in water solution (130 g per 2/4 liter of water) was prepared and in treatments of 1,2,3,4, diets were mixed with starch solution containing the meal of *Haematococcus pluvialis* algaein amounts of 25, 50, 75 and 100ppm astaxanthin respectively. In the control treatment, food wasn't prepared with the algae meal. Then foods were dried in an environment equipped with suitable ventilation.

Sampling and analysis methods

At the end of feeding period, 12 fish of each treatment (4fish from each pool) were sampled. Immediately after death of fish, three referees using Roche color fan (Roche SalmoFan™, Hoffmann-La Roche, and Basel, Switzerland) scored the color of back muscle of fish (20-34). Because the scores of back muscle of fish were below 20, for conducting a statistical analysis, all control treatment fish were given 19, then 9 of these fish (3 fish from each pool) were selected randomly and color of their back muscle was evaluated using a Colorimeter (HunterLab, D-25 DP-9000 model, Virginia, America). The measurements were performed in three repetitions within the colorimeter space of L*a*b* in which L* signify color brightness (100= completely white, 0=completely black; a*=red chromaticity index, b*= yellow chromaticity index). Then the measurements changed into the colorimeter space of L*C*H $(°)_{ab}$. In this space, the features of color vision include lightness (L*), hue H $(°)_{ab}$, and Chroma (C*).Chroma was calculated as $(a^{*2} + b^{*2)1/2}$ and hue calculated as arctan b*/a*. In order to measure the concentration of carotenoid, after removing skin of the 9 fish from each treatment, muscle above lateral line in the back area of fish was cut. After removing fat and bones, they became homogenous in a mixture (National model).Carotenoid was extracted using chloroform: methanol, based on Bilgh and Dyer method in 1959, with minor changes, in such a way that 10 g of the sample was mixed with 14 ml distilled water in a 250ml centrifuge bottle. 50 ml methanol was added (Merck Company) and the sample was mixed using a centrifuge (Beckman, L5-50 model, Lincoln, Nebraska, America) 2200rpm for 30 s. Then 25 ml chloroform (MerkCompany) was added and mixed for 30s. Finally, 25 ml distilled water was added and mixed for 30s, and the sample was centrifuged with 2900 rpm, in 10°c for 10 minutes. After centrifuge, methanol phase was separated,

protein layer translocate and chloroform phase containing carotenoids was separated. The protein layer again was extracted using the same method. The two chloroform phases were combined. Chloroform phase was protected against light by Aluminum foil packaging. Determination of total amount of carotenoids was performed using a spectrophotometer (UV-Vis spectrophotometer, Varian Company, Cary 100 Bio model, Victoria, Australia). All assays were repeated three times.

A reservation solution (100 mg/l) was prepared from pure astaxanthin (A9335, Sigma Company, St. Louis, Missouri, America) in chloroform (Merk Company), was packed in Aluminum foil and kept in 4°c. With diluting the reservation solution, standard solutions in concentrations of 20, and 5 mg/l were prepared. With diluting the 20 mg/l solution, more diluted standard solutions (4, 3, 2, 1 and 0/5 mg/l) were prepared. Using 1, 5 mg/l solutions, two spectrums of astaxanthin were measured in chloroform. Maximum absorbance obtained in wavelength of 487 nm for these spectrums (one of measurement units equal to 10^{-9}) (figure 1).

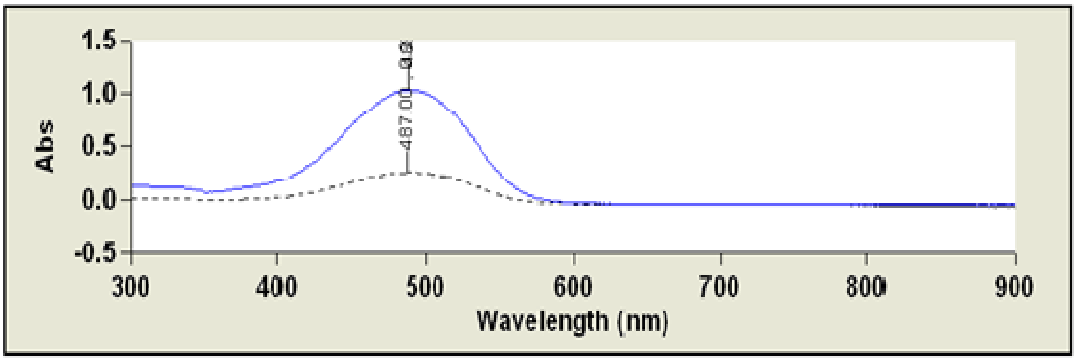

Figure 1: absorption spectrums of pure astaxanthin (concentration of 1, 5 mg/l) in chloroform indicate the maximum absorption in 487 nm. The solid line indicates 5mg/l; dashed line indicates 1 mg/l

The below standardcurve was prepared with solutions of 0, 0/5, 1, 1/5, 2, 3 and 4 mg/l(figure 2).

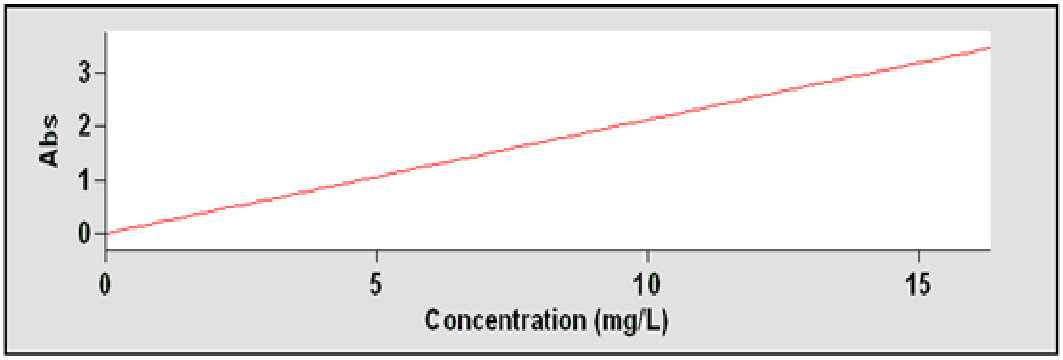

Figure 2: standard curve of pure astaxanthin (0, 0/5, 1, 2, 3, 4 mg/l) in chloroform along with Regression equation: R^2=0.25; y=0/2125x (measurement exactness representative)

Extinction coefficient was obtained from the slope of standard curve of linear regression. The extinction coefficient of astaxanthin in chloroform was$E_{1cm}^{1\%} = 2125$. Therefore, total amount of carotenoids in samples equaled astaxanthin (mg astaxanthin per 1 kg).

Data analyzed by SPSS (version 9/5), and the One-way ANOVA statistical method, Student-Newman-Keuls test (S-N-K) were used (P<0/05).

RESULTS

Prepared diets with different amounts of meal of *Haematococcus pluvialis* algae were accepted by fish and no mortality was observed. At the end of the test, no significant difference was observed for fish with different diets from the aspect of final weight (total mean of 358/56) and special growth rate (total mean of 1/02). Scores of color of fish muscle of Rainbow trout fed with diets containing different amounts of this alga is shown in figure 3.

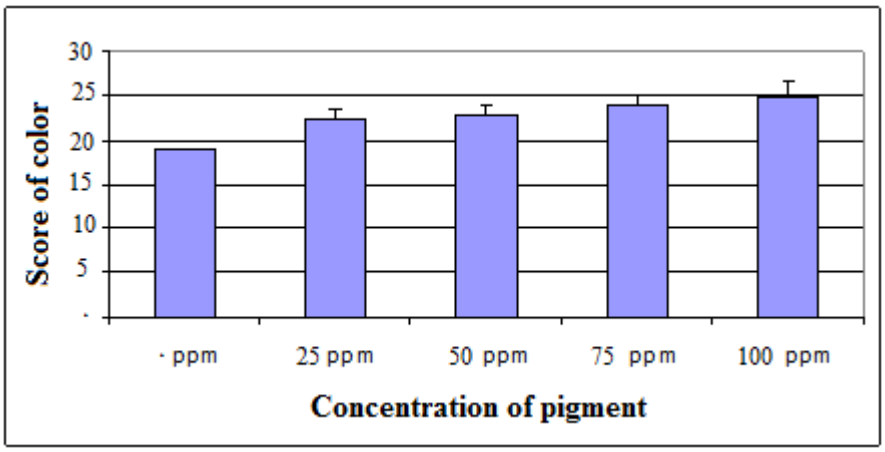

Figure 3: scores of color of fish muscle of Rainbow trout diagrams, mean values and small antennas indicate standard deviation

According to the figure 3, with increase of meal of *Haematococcus pluvialis* algae and therefore astaxanthin, the scores of color of fish muscles got increased. The scores of fish muscle color of each treatment was significantly different with that of fish from other treatments (p<0/01). Fish of treatment 2, fed with diets containing 50 ppm astaxanthin, had higher scores of muscle color compared to fish of treatment 1 which fed with diet containing 25ppm astaxanthin (p=0/019).
Indices of color of fish muscle of Rainbow trout fed with diets containing different amounts of the meal of *Haematococcus pluvialis* algae is shown in table 1.

Table 1: indices of color of fish muscle of Rainbow trout (1)
Means on one line with different letters are significantly different (P<0/05) (with S-N-K test)

Nutritional treatment by pigment concentration (ppm)	Color index				
	L*	C*	H(°)ab	a*	b*
0	76/88 (2/14)a	14/07 (2/33)a	79/62 (3/53)a	2/62 (1/13)a	13/80 (2/20)a
25	74/14 (1/90)b	21/50 (3/13)b	65/93 (5/62)b	8/93 (2/98)b	19/45 (2/29)b
50	73/38 (2/48)bc	22/73 (3/18)b	65/48 (4/26)b	9/56 (2/62)b	20/56 (2/45)bc
75	71/82 (3/10)c	23/65 (3/81)b	63/94 (4/93)bc	10/59 (3/25)b	21/06 (2/78)c
100	68/09 (5/34)d	25/96 (3/08)c	61/71 (3/32)c	12/36 (2/54)c	22/78 (2/27)d

As in the table 1, we can see with increase of this alga and, as the result, increase of astaxanthin in diet, the Chroma indices of C*, a* and b* have increased while lightness L* and hue H (°) $_{ab}$ has decreased. Fish of treatment 4, except for H (°) $_{ab}$ index, had significant difference with fish of other treatments from the aspects of all values of muscle color, in such a way that based on hue, there wasn't any significant difference between fish of treatments 3 and 4 (p> 0/05). Concentrations of carotenoid pigment of muscle of Rainbow trout fed with diets containing different amounts of the meal of *Haematococcus pluvialis* algae is offered in table 2.

Table 2: values of carotenoid pigment in muscle of Rainbow trout(1)

Pigment concentration in flesh (ppm)	Pigment concentration in diet (ppm)
0.28 (0.013)	0
1.22 (0.085)	25
1.54 (0.075)	50
1.87 (0.092)	75
2.46 (0.164)	100

(1) mean and standard deviation

According to the table 2, we find with increase of rate of the meal of *Haematococcus pluvialis* algae and, therefore astaxanthin in diet of fish, concentration of carotenoid pigment gets increase in fish muscle. The total amount of the obtained carotenoid for fish muscle of each treatment was significant compared to that of fish of other treatments (p<0/01). For example, pigment concentration in the muscle of fish of treatment 4 was 32 percent more than that of fish of treatment 3. Fish of treatment 2 compared to fish of treatment 3, had less pigment concentration in their muscle (p=0/019).
There was significant correlation between the scores of flesh color, all indices of the measured color and concentration of carotenoid in fish flesh (p<0/01).

DISCUSSION

Results shown in the table 1, is consistent with findings of Choubert *et al* [10] in which physical colorimeter showed that the added pigmentation of Rainbrow trout muscle lead to increase of Chroma and decrease of hue and lightness. This study affirmed results of tests conducted by Choubert *et al*. in 2006 which indicated increase of indices of C*, a* and b* in fish muscle of Rainbow trout during test of the receiver groups of pigment supplement obtained from *Haematococcus pluvialis* algae [10].
In texts related to the concentration of flesh pigment, there isa wide range of values. Cartenoid concentration for Rainbrow trout fish with weight range of 0/1-0/5 can reach to 6-7 mg/kg [7]. In this study, the most amount of pigment value was observed for one of samples related to treatment 4 which fed with diet containing 100ppm astaxanthin.
While conducting the first stage of extraction, major part of flesh pigment was retaken, visual observance showed that protein layer has kept a part of pigments. Therefore, conducting the second stage, retake of carotenoids was completed. In this study, the extinction coefficient was within 1700-2300, as was reported by Chen and Meyers in 1984 for different organic solvents. In addition, the absorption spectrum of astaxanthin in chloroform showed a single main message.
Results shown in the table 2, affirmed the results of regression analysis conducted by Sommer [8, 9] described significant correlation between the total values of carotenoids and astaxanthin in flesh of Rainbow trout and algae pigment in diet of fish. So, according to the present study we can conclude that the meal of *Haematococcus pluvialis* algae leads to increase of red color of fish flesh and is regarded an effective pigment source for it.
Depending water temperature, other important environmental factors and desired value of flesh color, addition of the meal of *Haematococcus pluvialis* algae in values of 75 or 100 ppm of astaxanthin to the diet of Rainbow trout fish is recommended.

ACKNOWLEDGMENTS

We would like to thank Cyanotech for sending the needed meal of *Haematococcus pluvialis* algae, management of Jajrood workshop for providing needed facilities and space for conducting this test and dear personnel's of the workshop for close collaboration, Miss Marjan Ghhotbi for providing us with the color fan, Sigma Company for sending us the standard astaxanthin, Dr. Riazi for honest collaboration for conducting this test and Dr. Negarestan for help with research design and conducting the statistical analysis.

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