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Research Article

# Pigmentation of Rainbow Trout (*Oncorhynchus mykiss*) with Carotenoids from Marigold Flower (*Tagetes erecta*) and Red Pepper (*Capsicum annum*)

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**Abstract:** The effects of diets including various natural carotenoid sources and synthetic astaxanthin on the pigmentation and growth of rainbow trout (*Oncorhynchus mykiss*) were investigated.

Rainbow trout weighing about 111 g were fed diets containing 1.6%, 2.4% and 3.2% marigold flower; 4.4%, 6.6% and 8.8% red pepper; 100 mg $kg^{-1}$ synthetic astaxanthin; and a control diet for 60 days. At the end of the experiment, synthetic astaxanthin provided the highest carotenoid accumulation in the fish flesh (6.42 mg $kg^{-1}$) in the groups. This was followed by red pepper (5.78 mg $kg^{-1}$) and marigold flower (5.59 mg $kg^{-1}$), each of which included equal levels (100 mg $kg^{-1}$) of carotenoids ($P < 0.05$). The fish fed marigold flower experienced yellow pigmentation, which was remarkably different from the other groups. An addition level of 2.4% or higher marigold flower and 6.6% or higher red pepper into the diet had negative effects on growth performance ($P < 0.05$). It can be concluded that the most appropriate dietary doses of marigold flower and red pepper for pigmentation of rainbow trout are 1.6% and 4.4%, respectively.

Key Words: Pigmentation, *Oncorhynchus mykiss*, red pepper, marigold flower

## Kadife Çiçeği (*Tagetes erecta*) ve Kırmızı Biber (*Capsicum annum*)'de Bulunan Karotenoitlerle Gökkuşağı Alabalığı (*Oncorhynchus mykiss*)'nın Pigmentasyonu

**Özet:** Bu çalışmada, karotenoit içeren çeşitli bitkisel yem kaynakları ve sentetik astaksantinin gökkuşağı alabalığı (*Oncorhynchus mykiss*)'nın pigmentasyonu ve büyümesi üzerine olan etkileri araştırılmıştır.

Yaklaşık 111 g olan alabalıklar, % 1,6, 2,4 ve 3,2 oranlarında kadife çiçeği (*Tagetes erecta*), % 4,4, 6,6 ve 8,8 oranlarında kırmızı biber (*Capsicum annum*), 100 mg/kg sentetik astaksantin ve kontrol grubundan oluşan diyetlerle 60 gün boyunca beslenmiştir. Gruplar arasında, kas dokuda en yüksek karotenoit birikimini sentetik astaksantin (6,42 mg/kg) sağlarken, bunu eşdeğer total karotenoit içeren (100 mg/kg) kırmızı biber (5,78 mg/kg) ve kadife çiçeği (5,59 mg/kg) izlemiştir ($P < 0,05$). Kadife çiçeği katkılı dietlerle beslenen balıklarda, diğer gruplardan farklı olarak sarı renklenme oluşmuştur. Diyetlere, kadife çiçeğinin % 2,4'e, kırmızı biberin ise % 6,6'ya eşdeğer veya daha fazla oranda katılması balıklarda büyümeyi olumsuz etkilemiştir ($P < 0,05$). Gökkuşağı alabalığının pigmentasyonu için kadife çiçeği ve kırmızı biberin diyetteki en uygun düzeylerinin sırasıyla % 1,6 ve % 4,4 olduğu önerilir.

Anahtar Sözcükler: Pigmentasyon, *Oncorhynchus mykiss*, kırmızı biber, kadife çiçeği

## Introduction

Fish nutrition has an important impact on several parameters directly influencing the quality of fish, such as colour and appearance. The colour of fish fillets is one of the most important quality parameters (1) because consumers tend to prefer red or pink products of salmonid fishes (2-4). Therefore, it plays a decisive role when evaluating the quality of the product at point-of-sale.

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Carotenoids, especially astaxanthin, are responsible for the typical colour of salmonid muscle (5,6). In addition, they act as antioxidants (7,8) and precursors of vitamin A (9,10). Fish are not able to perform de novo the synthesis of carotenoids (11), and they must obtain them via their diet. The carotenoids can be produced synthetically and are commonly used for pigmentation of salmonids; however, alternative natural carotenoid sources have also been studied because of public concerns about the use of synthetic additives.

Recently, the most promising alternatives to synthetic astaxanthin for salmonids pigmentation are the red yeast *Phaffia rhodozyma* (12,13), *Haematococcus algae* (14,15), *Longostilla*, *Pleuroncodes planipes* (16) and *Chlorella vulgaris* (17). In addition, red pepper (*Capsicum annum*) and marigold flower (*Tagetes erecta*), which are abundant and rich in carotenoid pigments, could be considered alternative sources. It was shown that red pepper could be used for pigmentation of salmonids (18,19). However, it is still not certain what percentage of red pepper can be used in rainbow trout diets or what effects it has on growth. To the best of our knowledge, no investigations on marigold flower have been published.

In the present study, the effects of diets including various proportions of red pepper and marigold flower on the pigmentation of rainbow trout were examined in comparison to synthetic astaxanthin as well as their potential as additives in rainbow trout diets.

## Materials and Methods

### Experimental procedure and feeding trial

The experiment was conducted in floating net cages (1 x 1 x 1 m), for 60 days, in the Sir Dam Lake, Kahramanmaraş, Turkey. Thirty rainbow trout, *Oncorhynchus mykiss*, each of which were around 110-115 g in weight, were stocked in separate cages as 2 replicates for each experimental feed. The fish were fed ad libitum 3 times on a daily basis. On days 0, 20, 40 and 60, all fish remaining in each cage were weighed prior to sampling for measurement of total carotenoids. Throughout the experiment, the temperature, dissolved oxygen level and pH of the rearing water were 7.95-8.26 °C, 9.99-10.73 mg $l^{-1}$ and 7.24-7.35, respectively. The 8 different pelleted feeds used in the experiment were as follows:

-Diet supplemented with 4.4% red pepper (containing 65 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 6.6% red pepper (containing 100 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 8.8% red pepper (containing 130 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 1.2% marigold flower (containing 65 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 2.4% marigold flower (containing 100 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 3.6% marigold flower (containing 130 mg $kg^{-1}$ total carotenoid)

-Diet supplemented with 100 mg $kg^{-1}$ synthetic astaxanthin

-Diet without carotenoid (control or basal diet).

The commercial basal diet was supplied by Çamlı A.Ş, İzmir, Turkey (Table 1), synthetic astaxanthin, in the form of carophyll pink (Hoffman La Roche, Switzerland), whereas the red pepper and marigold flower were provided by local producers. Both plants were dried in the shade, ground into meal, and added to the food mixture in different proportions. Carophyll pink was dissolved in water at 60 °C prior to adding to the pelleted feed at 100 mg $kg^{-1}$. (To moderate the protein imbalance occurring due to plant additives between the diet groups, fish-meal including 65% protein was added as appropriate.)

Table 1. Nutritional composition of control diet (basal diet). Data obtained from Pınar A.Ş., Turkey.

| Chemical composition | Percentage (%) |
|---|---|
| Crude protein | 44.5 |
| Crude fat | 20.1 |
| Crude cellulose | 2,89 |
| Crude ash | 13 |
| Dry matter | 88 |
| Energy content (ME kg/cal) | 4100 |
| *Vitamin and mineral content | |

* Supplied per kilogram of feed: 25,000 i.u. vitamin A; 2000 i.u. vitamin D3; 100 i.u. vitamin E; 150 mg vitamin C; 15 mg vitamin K; 20 mg thiamine; 30 mg vitamin B2; 10 mg panthothenic acid; 20 mg pyridoxin; 210 mg inositol; 2000 mg choline; 0.05 mg vitamin B12; 220 mg niacin; 0.5 mg biotin; 5.0 mg folic acid; 70 mg Zn; 60 mg Mg; 4.0 mg Fe; 20 mg Cu; 0.5 mg Co; 0.05 mg Se

The pellets for all 8 diets were produced in the same way. The ingredients were first turned into a homogeneous doughy consistency by adding water, and converted into pellet form by being pressed through a sieve with 4 mm holes in a grinding machine. The pellets were stored in refrigerator containers at -20 °C. They were thawed before they were given to the fish (19).

### Proximate composition analysis

Lipids were extracted as described by Bligh and Dyer (20). Ash and moisture contents were determined according to AOAC (21) and crude protein content was calculated by converting the nitrogen content determined by Kjeldahl's method (6.25 x N) (21), and crude cellulose was determined by the method of AOAC (21) (Table 2).

### Carotenoid analysis

Dissection of the fish and extraction of carotenoids were carried out as described by Torrissen and Naevdal (22): 4 fish taken from each diet treatment were used for each carotenoid analysis, and these analyses were carried out in duplicate. The fish were filleted by removing the flesh between the pelvis and anus on both sides of the animal, and the skin was subsequently removed. The removed muscle samples were minced with a meat mincer, and samples of approximately 1-2 g were taken for analysis, and then transferred to 10-ml pre-weighed glass tubes.

First, 10 ml of dry acetone and then about 2 g of anhydrous sodium sulphate were added to the samples. The solutions were centrifuged at 5000 rpm for 5 min, and then stored at 4 °C in a refrigerator. After 3 days of extraction in sealed glass tubes, the absorption of the extracts was measured at 476 nm in a spectrophotometer. A similar method was used for the total carotenoid analysis of red pepper and marigold flower meal, but anhydrous sodium sulphate was not used in these plants as it was already in dry form, and the solution was measured at 450 nm.

Total carotenoid concentration in the fish fillet was determined spectrophotometerically in acetone using $E_{(1\%, 1cm)}$ = 1900 (23) at 474 nm and for both plant meals, $E_{(1\%, 1cm)}$ = 2500 (24) at 450 nm. The total carotenoid concentrations of dried red pepper and marigold flower meal were determined as 1500 and 4200 mg $kg^{-1}$, respectively, and these amounts were taken into account while adding to diets (Table 2).

### Statistical analysis

Growth data and pigment levels of the fish taken separately from each treatment diet for each sampling time (on day 0, 20, 40 and 60) were analysed by analysis of variance (one-way ANOVA), and the significant differences in ANOVA were ranked with Duncan's multiple comparison test at the 5% level of significance in SPSS.

## Results

Growth data of the fish on days 0, 20, 40 and 60 are summarised in Table 3. There were no significant differences in the weight gains of the fish on day 20 ($P > 0.05$), but growth differed significantly among the groups on days 40 and 60 ($P < 0.05$). On these sampling days, the highest weight gain was displayed by the control group and synthetic astaxanthin and 4.4% red pepper, followed by the groups of 1.6% marigold flower, 6.6% and 8.8% red pepper, and 2.4% and 3.2% marigold flower ($P < 0.05$).

Total carotenoid contents in the fish muscle are presented in Table 4. Diet groups were significantly different for carotenoid contents in fish muscle on all sampling days ($P < 0.05$). Carotenoid contents of the fish fed each diet supplemented with a pigment source were significantly higher than those of the control group. At the onset of the experiment, the carotenoid level in the

Table 2. Proximate composition and carotenoid contents of marigold flower and red pepper used in the experiment.

| Carotenoid sources | Crude protein % | Crude fat % | Crude cellulose % | Carotenoid content (mg $kg^{-1}$) |
|---|---|---|---|---|
| Marigold flower | 12.22 | 9.20 | 14.3 | 4200 |
| Red pepper | 12.32 | 16.13 | 21.10 | 1500 |

Table 3. Mean weight of the fish fed various diets.

| Diets | Mean weight of the fish (g) | | | |
|---|---|---|---|---|
| | day 0 | day 20 | day 40 | day 60 |
| 1.6% marigold flower | 113.99 ± 1.17 [a] | 140.61 ± 1.27 [a] | 172.60 ± 1.80 [abc] | 215.06 ± 2.73 [abc] |
| 2.4% marigold flower | 112.98 ± 1.20 [a] | 139.71 ± 1.34 [a] | 168.75 ± 1.86 [c] | 210.01 ± 2.74 [c] |
| 3.2% marigold flower | 111.33 ± 1.16 [a] | 139.53 ± 1.49 [a] | 168.25 ± 2.11 [c] | 209.03 ± 3.32 [c] |
| 4.4% red pepper | 114.39 ± 1.27 [a] | 142.76 ± 1.49 [a] | 176.02 ± 2.25 [abc] | 220.63 ± 3.82 [abc] |
| 6.6% red pepper | 109.91 ± 1.11 [a] | 138.31 ± 1.56 [a] | 170.67 ± 1.95 [bc] | 214.65 ± 2.67 [bc] |
| 8.8% red pepper | 109.81 ± 1.20 [a] | 138.01 ± 1.72 [a] | 170.01 ± 2.05 [bc] | 213.31 ± 2.75 [bc] |
| 100 mg $kg^{-1}$ astaxanthin | 110.71 ± 1.11 [a] | 142.74 ± 1.59 [a] | 178.71 ± 2.42 [ab] | 227.49 ± 3.09 [ab] |
| Control | 110.92 ± 1.20 [a] | 143.71 ± 1.51 [a] | 179.91 ± 1.99 [a] | 228.11 ± 2.70 [a] |

Each value is a mean ± s.e. (n = 2). Each replicate consists of measurements from 30 fish.
The means with different letters in each column denote a significant difference (P < 0.05)

Table 4. Total carotenoid contents in the fillet of the fish fed various diets.

| Diets | Total carotenoid contents (mg $kg^{-1}$) on sampling day | | | |
|---|---|---|---|---|
| | day 0 | day 20 | day 40 | day 60 |
| 1.6% marigold flower | 0.820 ± 0.005 [a] | 1.773 ± 0.021 [f] | 3.181 ± 0.039 [f] | 4.480 ± 0.040 [g] |
| 2.4% marigold flower | 0.820 ± 0.005 [a] | 2.206 ± 0.019 [e] | 4.361 ± 0.033 [d] | 5.590 ± 0.057 [e] |
| 3.2% marigold flower | 0.820 ± 0.005 [a] | 2.585 ± 0.071 [c] | 5.186 ± 0.023 [c] | 6.168 ± 0.029 [c] |
| 4.4% red pepper | 0.820 ± 0.005 [a] | 2.163 ± 0.059 [e] | 3.375 ± 0.056 [e] | 4.555 ± 0.028 [f] |
| 6.6% red pepper | 0.820 ± 0.005 [a] | 2.435 ± 0.024 [d] | 4.438 ± 0.020 [d] | 5.783 ± 0.026 [d] |
| 8.8% red pepper | 0.820 ± 0.005 [a] | 2.806 ± 0.033 [b] | 5.313 ± 0.077 [b] | 6.370 ± 0.020 [b] |
| 100 mg kg-1 astaxanthin | 0.820 ± 0.005 [a] | 2.943 ± 0.026 [a] | 5.668 ± 0.049 [a] | 6.421 ± 0.020 [a] |
| Control | 0.820 ± 0.005 [a] | 1.053 ± 0.079 [g] | 1.025 ± 0.079 [g] | 1.236 ± 0.025 [h] |

Each value is a mean ± s.e. (n = 2). Each replicate consists of measurements from 4 fish.
The means with different letters in each column denote a significant difference (P < 0.05)

fillets was 0.82 mg $kg^{-1}$; this value had risen to 4.4 mg $kg^{-1}$ (with 2.4% marigold flower) and 6.42 mg $kg^{-1}$ (with synthetic astaxanthin) by the end of the experiment. On the other hand, carotenoid content was fairly similar or nearly stable (1.23 mg $kg^{-1}$) throughout the study in the control group. Although the synthetic astaxanthin included less carotenoid content (100 mg $kg^{-1}$) compared to 8% red pepper and 2.4% marigold flower meals (including 130 mg $kg^{-1}$ total carotenoid each), the carotenoid content in the fish muscle was higher than that in the fish fed red pepper and marigold flower. This was followed by red pepper and marigold flower.

## Discussion

Beginning from day 40, the diets supplemented with levels equal to or over 6.6% red pepper or 2.4% marigold flower had a negative effect on the growth of the fish. Several studies show (25-27) that the use of high levels of plant material in diets of fish, especially carnivorous fish, retards their growth. The main reason for this has been considered due to containing high level cellulose of the plant. In addition, they might lead to negative effects on the taste of food, physical quality of the pellets and nutrition balance of diets (28). However, the degree of this effect naturally depends on the feeding habit of the fish and the preparation of the diet. Rainbow trout is a carnivorous fish and, therefore, adding plants to their diets will be naturally restricted.

The addition of red pepper and marigold flower at each level increased the pigmentation level, even within 20 days. Although a significant difference in carotenoid accumulation appeared between the groups including the high dose of carotenoids until day 20, this difference had decreased notably by day 60. However, in the groups including the lower dose of carotenoid, carotenoid accumulation rates were nearly stable throughout the culture period (Table 4). The fact that the muscle tissue reaches its maximum carotenoid accumulation level (satiation point) is thought to be the reason for this situation. In fact, the satiation point for rainbow trout fillet was found to be approximately between 6 and 8 mg $kg^{-1}$ (29,30). The maximum level of carotenoid concentration (6.16-6.37 mg $kg^{-1}$) obtained in our study was within this range.

A level of 4 mg $kg^{-1}$ in the fish fillet is regarded as a minimum acceptable carotenoid concentration in marketable farmed salmon (30). In our study, even though synthetic astaxanthin provided better pigmentation than did red pepper or marigold flower (similar results were also reported on red pepper by Carter et al. (18) and Yanar et al. (19)), even the lowest level of carotenoid (4.48-4.55 mg $kg^{-1}$) provided with 4.4% red pepper and 1.6% marigold flower was found to be adequate for a desired colouration in rainbow trout. Furthermore, these levels of red pepper or marigold flower did not interfere with growth.

The synthetic astaxanthin was found to be more effective than red pepper and marigold flower although they contained equal amounts of carotenoids. The reason for this may be differences in carotenoid sources. Although salmonids absorb astaxanthin 10-20 times as much as lutein and zeaxanthin (30), in our study, the lutein from marigold petal was absorbed to such an extent that it was not negligible (Table 4). Moreover, although it is stated that various carotenoids are deposited in salmon fillet as astaxanthin, which is responsible for the typical pink colour of salmonids muscle (5,6), in our study the yellow pigmentation occurred in the fillets, which shows that the dietary carotenoids might have been deposited in the fish partly as lutein or other xanthophylls form characterised by the yellow colour. However, the yellow pigmentation formed in the fish fed with marigold flower may not be desirable by consumers. Therefore, consumer preferences should be taken into consideration.

In conclusion, synthetic astaxanthin provided the highest carotenoid accumulation in the flesh among the groups. This was followed by red pepper and marigold flower. The yellow pigmentation formed in the fillet of the fish fed marigold flower was distinctly different from that of the other groups. An additive level of equal to or higher than 2.4% marigold flower or 6.6% red pepper in the diet had a negative effect on the growth performance of the fish. It can be concluded that the most appropriate doses of marigold flower and red pepper in the diet for pigmentation of rainbow trout are 1.6% and 4.4%, respectively.

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