Carotenoid pigment content and skin microscopic appearance of cultured Ajamaru Rainbowfish (*Melanotaenia ajamaruensis*)

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Abstract. The color of Ajamaru Rainbowfish (*Melanotaenia ajamaruensis),* which is adapted to the tank culture tends to decrease in brightness and become dull. It is necessary to improve the quality of color, especially through the diet. This study was conducted to determine the effect of the supplementation of carotenoid on the diet on the amount of carotenoid pigments and the microscopic appearance of the skin, fins, and scales of the Ajamaru rainbowfish. The research used a factorial design, consisting of two factors and three replications. The Factors were three different diets (carotenoid-free diet, astaxanthin, and lutein diet) as factor 1, and gender (male and female) as factor 2. The observed data were carotenoid content in fins, skin, and flesh; the microscopic appearance of fins and scales was measured at the end of the study. The results showed that the supplementation of astaxanthin and lutein to the feed increased the content of carotenoid pigments in the fins and scales of the Ajamaru rainbowfish; the highest values in the fins and skin of the male fish were 202.90 and 8.53 ppm, respectively. The effect of astaxanthin and lutein supplementation on color can be observed microscopically in the tissue of scales and fins.

1 Introduction

Ajamaru rainbowfish (*Melanotaenia ajamaruensis*) is one of Indonesia's endemic freshwater fish, which is only found in Ajamaru Lakes, Papua. *M. ajamaruensis* has a metallic blue to yellowish or green with orange and yellow longitudinal stripes and dark scale edges in its natural habitat. The body is ovate and laterally compressed. Mature males have a higher first dorsal fin [1]. The male fish is orange to red, while the color of the female fish is yellow to orange [2]. The color of cultured fish was not as bright as the color of naturally captured fish. Its color becomes dull because of the feed and environment container. The quality of color in ornamental fish influences the market price [3], therefore it is necessary to improve the quality of color in rearing of Ajamaru rainbowfish.

The quality of color improvement in ornamental fish can be reached by providing a diet containing carotenoids. It is necessary to provide astaxanthin, canthaxanthin and lutein in the

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diet, and astaxanthin-type carotenoids are the most effective in improving the color of *Melanotaenia parva* fish compared to other types. [4]. On the other hand, feed containing lutein had the same efficiency as astaxanthin and canthaxanthin for pigmentation in goldfish [5]. These carotenoids will fulfill, increase the number and enlarge the color cells called chromatophores, which is known as morphological color changes [6].

Two chromatophore cells contain carotenoids, namely erytrophore and xanthophore [7]. Erytrophore and xanthophore cells are scattered in different body tissues of fish, especially in the skin, scales, and fins. These cells can be observed by biopsy [8]. Rainbow ornamental fish are generally easily stressed and change color when placed in an aquarium [9], it is known as physiological color change [10], making it difficult to measure color using a colorimeter or digital images. Sampling and observing under a microscope are an accurate and simple approach compared to histology.

This study aimed to determine the effect of carotenoid supplementation on the diet to the number of carotenoid pigments and the microscopic appearance of the skin, fins, and scales of the *Melanotaenia ajamaruensis* fish, as an indication of the effect on color when fed long-term.

2 Material and methods

The study used Ajamaru rainbowfish (*Melanotaenia ajamaruensis*) which were 2-month-old fish obtained from the Research Institute for Ornamental Fish Culture. There were 90 test fish were divided into 18 aquariums (5 fishes in each aquarium) sized 30 x 20 x 20 cm3 with a water level of 13 cm. The fish, separated into males and females, were then given treatment diets, namely control (no carotenoid supplement), (A) diet supplemented with astaxanthin (*Carophyll Pink* 10% - DSM), and (B) diet supplemented with lutein (*Carophyll Yellow* 10% - DSM). Each treatment was replicated three times. The dietary test was conducted for 30 days. This study used a stagnant water system with the addition of an aerator to stabilize dissolved oxygen. Feed was given three times a day at satiation. Feed residues and feces are siphoned off daily to maintain stable water quality.

The composition of the feed formulation is shown in **Table 1**. The feed ingredients were analyzed immediately before formulation to obtain a feed that is iso-protein and iso-lipid. The composition of the fish feed formulation is based on the requirements of freshwater shrimp [11] with a protein content of 35% and a fat content of 8%. The feed was produced in the form of crumbles with a US Mash #30 size and stuck in US Mash #40.

Table 1. Eksperimental test feed composition

No	Feed Ingredients	Control	Astaxanthin	Lutein
1.	Fish meal (g)	285.5	285.5	285.5
2.	Soybean meal (g)	250	250	250
3.	Pollard (g)	200	200	200
4.	Wheat flour (g)	200	200	200
5.	Fish oil (g)	45	45	45
6.	Vitamin Mix (g)	10	10	10
7.	MDC Phosphate (g)	9.5	4.5	4.5
8.	Astaxanthin (g)	-	5	-
9.	Lutein (g)	-	-	5
Proximate composition (% dry matter)				
Protein		35.03	35.69	35.67

Fat	8.20	8.20	8.20
Crude fiber	3.65	3.65	3.65
Nitrogen free extract	46.07	45.41	45.43
Ash	7.05	7.05	7.05

Carotenoid content was observed in the fins, skin, and flesh. Additionally, colors were compared visually, and microscopic images of fins and scales were taken at the end of the study. The measurement of carotenoid pigments was performed according to [12] with some modifications. Each skin/fin/tail sample was taken up to 0.03-0.2 g with anhydrous sodium sulfate and placed in a test tube, then 5 ml of acetone (98%, Merck, Germany) was added and homogenized using Ultra Turrax T25. Homogeneous samples were stored at 4°C overnight and then extracted 2-3 times until colorless. The extracted solution was centrifuged at 3500 rpm for 10 min and then read using a spectrophotometer at 380, 450, 475, and 500 nm wavelengths. The carotenoid content was calculated from the highest absorbance value with a specific extinction coefficient (E1%, 1 cm) of 2500.

Carotenoid content data from the study results were analyzed using ANOVA and Duncan's tests were performed when significant differences were found. Microscopic appearance data were analyzed descriptively.

3 Result and discussion

The results showed that there were differences in the amount of carotenoid pigments in the body tissues of male fish compared to female fish. The supplementation of astaxanthin and lutein in the diet can increase the amount of pigment, especially in the fins. The detailed results of the research are shown in **Table 2**.

Table 2. Carotenoid pigment content of the skin, fin, and flesh of Ajamaru rainbowfish fed with free carotenoid, astaxanthin, and lutein

Sex	Treatments	Skin (ppm)	Fin (ppm)	Flesh (ppm)
Male	Control	2.14 ± 0.01^{b}	60.00 ± 2.73^{b}	0.43 ± 0.20^{a}
	Astaxanthin	8.53 ± 0.26^{d}	202.90 ± 3.08^{f}	2.29 ± 0.08^{b}
	Lutein	5.68 ± 0.04^{bc}	135.48 ± 1.02^{e}	4.86 ± 0.21^{c}
Female	Control	0.48 ± 0.05^{a}	9.47 ± 0.81^{a}	1.02 ± 0.05^{a}
	Astaxanthin	5.82 ± 0.09^{c}	96.88 ± 0.21^{c}	0.81 ± 0.08^{a}
	Lutein	5.88 ± 0.08^{c}	117.62 ± 1.68^{d}	0.89 ± 0.04^{a}

Note: the different superscripts in the same column indicate significant differences (P<0.05).

Statistical analysis showed that the carotenoid content of the skin and fins of male fish fed the control diet was higher than the pigment content of female fish, but the composition of the flesh was the same. Pigments in fish tissue represent substances that are emitted as color through the chromatophore cells, as described by [7] that color changes in fish occur through pigment cells called chromatophores. This suggests that the color of male fish is naturally lighter than female fish, as reported by [2] in the form of photographs of Ajamaru rainbowfish caught in the wild (**Figure 1 a-b**), but there are differences with the results of the study (**Figure 1 c-h**).

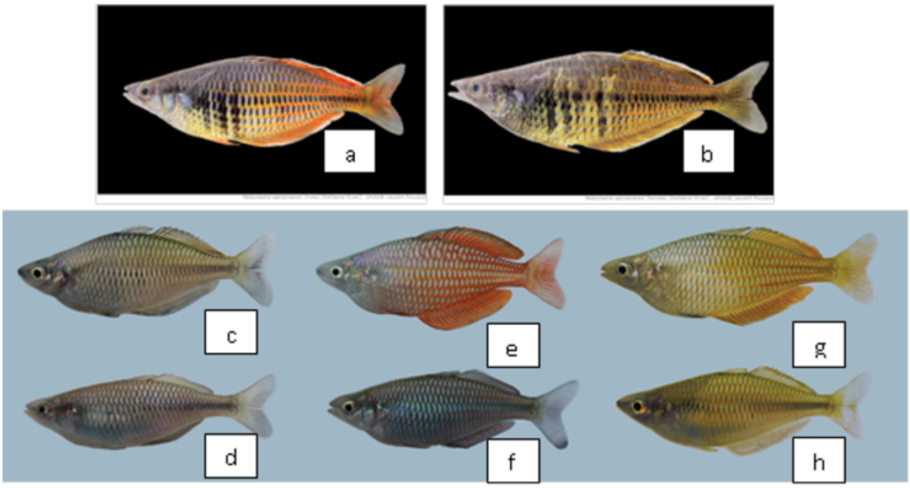


Fig. 1. a=male Ajamaru rainbowfish captured in the wild; b. female Ajamaru rainbowfish captured in the wild (source: [2]); c=male fish fed control diet; d=female fish fed control diet; e=male fish fed astaxanthin diet; f=female fish fed astaxanthin diet; g=male fish fed lutein diet; h=female fish fed lutein diet

In the wild, male fish have red fins and skin, while female fish have yellow coloring. However, the color of farmed fish is influenced by their diet. Male fish that are fed astaxanthin tend to have a redder color than those fed other types of feed. On the other hand, both male and female fish given lutein supplementation (**Figure 1g and 1h**) develop a yellow color. This color variation is due to the carotenoids present in the fish's diet and how their bodies store these pigments. **Figure 1e** displays the red coloring of male fish.

The results of this study showed that the supplementation of carotenoids in the form of astaxanthin to the diet resulted in the highest levels of carotenoid pigments in the fins and skin of male fish, namely 202.90 and 8.53 ppm, respectively. In female fish, the highest level of carotenoid pigments was obtained with the supplementation of lutein, namely in the fins at 117.62 ppm. It has been observed that female fish have the same levels of carotenoid pigments in their skin and flesh as those achieved through astaxanthin supplementation. This suggests that male and female fish exhibit different responses to the supplementation of two distinct types of carotenoids. This difference in response is anticipated because male and female Ajamaru rainbow fish possess unique abilities to utilize dietary carotenoids for the pigmentation process in their body tissues. Several factors influence the pigmentation process in fish and shrimp, including those that are internal to the animal itself, such as species, genetics, age/stadia, metabolism, sex, target tissue, and molting conditions [13]. Regarding the age factor of the fish, its relationship with pigmentation was reported by [14] that pigmentation in rainbow kurumoi (*Melanotaenia parva*) fish is microscopically visible from the eighth day after eggs hatch into larvae. According to [15] male fish generally have brighter colors than females, which serves to attract the attention of females. This is related to the level of the hormone testosterone in the fish's body, as reported by [16], who stated that administration of 17α-methyltestosterone gave male lemon fish a better color than females. On the other hand, there are external factors, one of which is the type and structure of the pigment given.

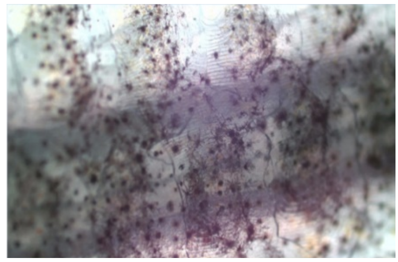
In this study, 2 types of carotenoids were given, namely astaxanthin and lutein. Astaxanthin is a type of fat-soluble carotenoid with a chemical structure of $C_{40}H_{52}O_4$, physically it has a red color [17], making it easier to deposit in the tissues of male Ajamaru rainbowfish. This pigment is likely to be deposited in the erythrocyte cells, as these cells are known to deposit carotenoids and emit orange to red colors [7,18]. Meanwhile, lutein is a pigment in the form of yellow crystals [19], which is common in freshwater fish [20, 21], so it is thought that it is more easily stored in the tissues of female fish, which are naturally yellow. This yellow pigment is stored in xanthophores cells, which are capable of storing yellow to orange carotenoids [7]. These results are also consistent with the [20] report that fish color is produced by the carotenoids astaxanthin (pink), canthaxanthin (red), lutein (yellow), beta-carotene (orange), and zeaxanthin (orange).

The results of this study are different from reports and results of other studies, which mainly classify carotenoid absorption only up to the grouping of fish species. In their publications, [17, 22] classified three groups of aquatic animals that use carotenoids, namely: (1) Crustacea group: Aquatic animals that are omnivorous, have a low body mass but a high ability to convert various carotenoids into astaxanthin stored in their carapace, including carotenoids in algae (lutein and zeaxanthin) and β-carotene; (2) groups of herbivorous fish, such as carp, which can store β-carotene, lutein, zeaxanthin, and canthaxanthin in their bodies without altering their original structure and can convert them into astaxanthin; (3) the group of carnivorous fish, which are only able to store lutein, zeaxanthin and canthaxanthin in their original form and are unable, or very poorly able, to convert these substances into astaxanthin. As reported by [4] dietary astaxanthin was most effective in increasing color and pigmentation in Kurumoi rainbowfish (*Melanotaenia parva*), implying that this species was only able to convert a small amount of pigment other than astaxanthin and store it in its body tissues. The report did not explain whether there were differences in the response of male and female fish to the intake of different carotenoid sources.

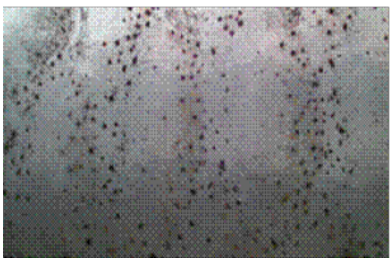
In this study, male Ajamaru rainbowfish (**Table 2**) were more responsive to astaxanthin, while female fish were more responsive to lutein. When comparing research results to previous publications, differences may arise due to advancements in scientific knowledge or variances in the species being tested. This information can be useful in determining the appropriate pigment to use when formulating a feed for ornamental rainbowfish. The accuracy of these findings has been validated through the microscopic examination of color images on the scales and fins of the fish. When comparing microscopic analysis of the Ajamaru rainbowfish to histological analysis, it was found that the latter provided a more detailed description of chromatophore cells. However, histology on this type of fish can be challenging due to the complexity of color distribution and difficulty in determining which part to analyze. This is different from salmon which has a more uniform distribution of colors and is dominated by melanophore cells on the skin [23]. The microscopic analysis approach was carried out as an early indication of the effect of giving carotenoid pigments on the color of the Ajamaru rainbow fish, where the color change can occur in a short time. A description of the color of the Ajamaru rainbowfish has been submitted by [2] in general, male fish are orange to red, with a uniform pattern of alternating red and orange stripes, the color of female fish is darker with a yellow-orange stripe pattern.

Ajamaru rainbowfish have a similar color to other rainbowfish, but their color can quickly change based on environmental and physiological factors. This is known as physiological color change, making it challenging to analyze their color accurately. However, a reliable method for analysis is presented in the theory by [24], which involves observing morphological discoloration by examining chromatophore cells using a microscope and the skin biopsy method [8]. The study found that all scales had black spots, particularly the anterior scales, compared to the fins.When comparing males and females, the black dots are more visible in females. The black dots visible on the scales and fins are thought to be melanophore cells. According to [6,7], melanophores are black to brown colored chromatophore cells with melanosome organelles, containing melanin pigment in the cytoplasm, and cell sizes ranging from 10 μm to 100 μm [23]. The difference in the number of male and female melanophore cells is due to the sexual status of the fish [8]. The results of the microscopic observations are shown in **Figure 2-4**

a. Front scales of male fish fed control diet



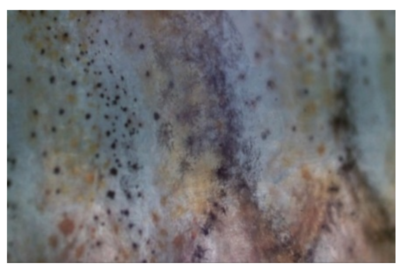
b. Middle scales of male fish fed the control diet



c. Front scales of male fish fed astaxanthin diet

d. Middle scales of male fish fed astaxanthin diet

e. Front scales of male fish fed lutein diet

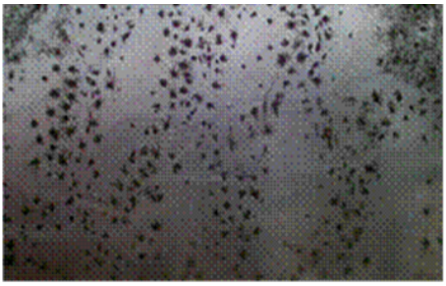


f. Middle scales of male fish fed lutein diet

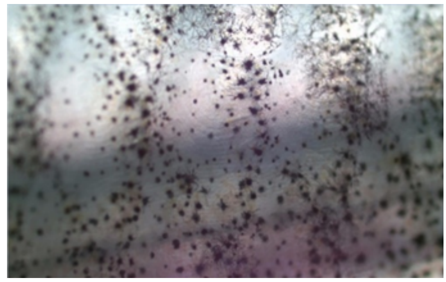
Figure 2. Results of microscopic observations on the scales of male rainbow ajamaru fish fed different diets

Figure 2 shows that there is an effect of supplementation of carotenoid pigments in the form of astaxanthin and lutein on the color of the scales of male Ajamaru rainbowfish. This can be seen from the enhancement of red color to the scales of male fish treated with astaxanthin (**Figures 2c-2d**) and yellow color to the scales of male fish treated with lutein (**Figures 2e - 2f**). This is consistent with [25] finding that carotenoids directly impart color to the erytrophore and xantophore cells. On the other hand, the fish on the control diet did not show any color change because they did not get enough carotenoids from their diet. This condition is a logical consequence because animals, including fish, are unable to produce carotenoids in their bodies and can only get them from the food they are given [6].

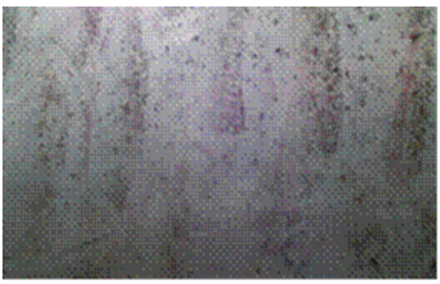
The female fish (shown in **Figure 3a-3f**) have a slightly lighter scale color compared to the male fish, which is likely due to having fewer chromatophore cells. Chromatophore cells are responsible for expressing color in fish, and generally, male fish have lighter colors than female fish, as explained by [10]. Additionally, [7] suggests that attractive colors in male fish can be used as a signal to attract female fish and indicate their dominance status. Therefore, when astaxanthin and lutein are added to female fish, the results are not as noticeable as in male fish. A microscopic image of female fish scales is presented in **Figure 3**.



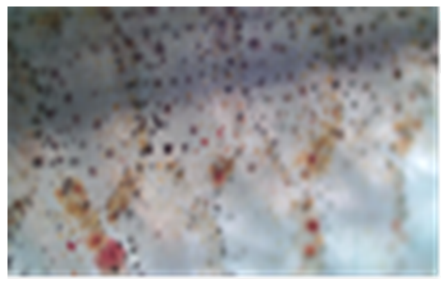
a. Front scales of female fish fed control diet



b. Middle scales of female fish fed control diet

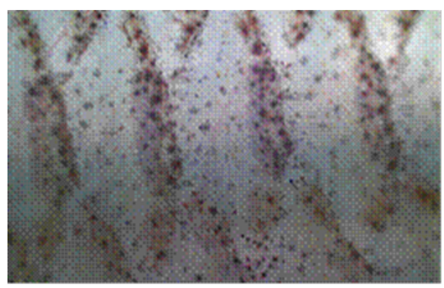


c. Front scales of female fish fed astaxanthin diet



d. Middle scales of female fish fed astaxanthin diet

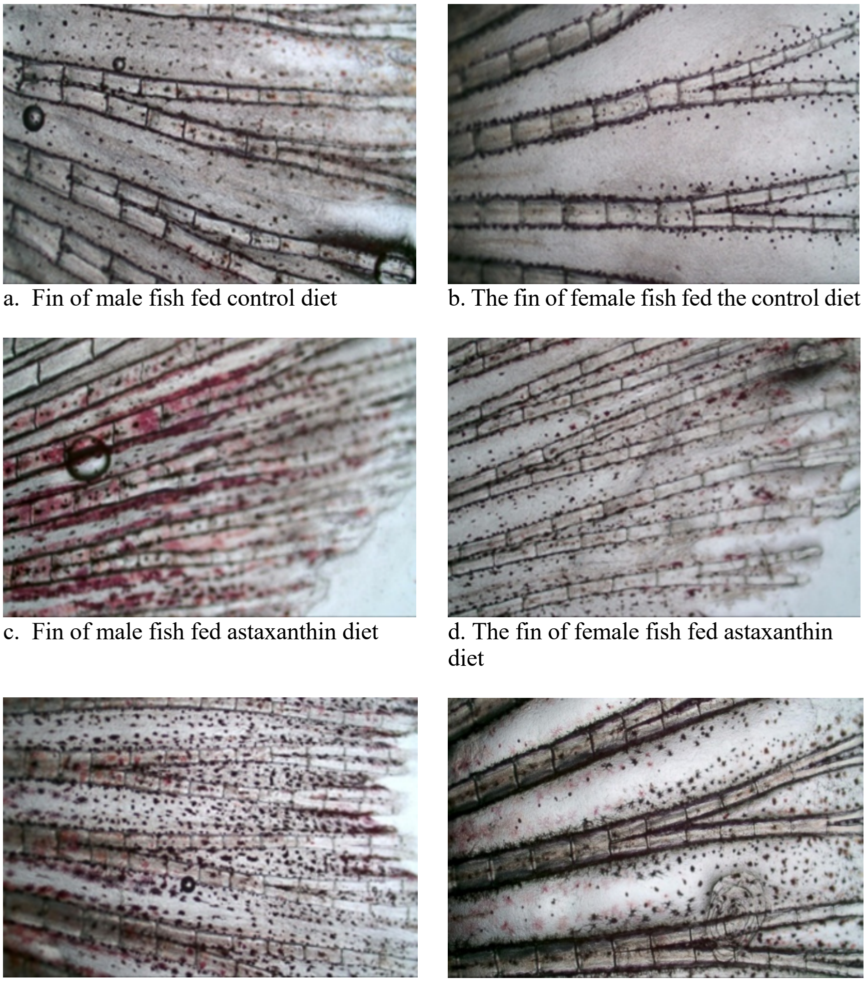
e. Front scales of female fish fed lutein diet



f. Middle scales of female fish fed lutein diet

Figure 3. Results of microscopic observations on the scales of female rainbow ajamaru fish fed different diets

In contrast to the microscopic appearance of the fins of the Ajamaru rainbowfish, the results showed that astaxanthin had a better effect on fin color compared to the control and the supplementation of lutein (**Figure 4**). This is in line with the opinion of [26] who stated that in some fish species, especially salmon, the absorption of astaxanthin is 10-20 times better than that of lutein and zeaxanthin. [27] then added that the absorption of astaxanthin in the body of goldfish is faster than the absorption of lutein and carotene. In **Figure 4c-4d** astaxanthin increased the number of red spots, whereas lutein administration to male Ajamaru rainbow fish decreased the number of red spots (**Figure 4e**) and resulted in yellow spots in female fish (**Figure 4f**). This is because astaxanthin has a pink base [28,29], whereas lutein is a natural carotenoid in the form of a yellow solid crystal [19]. Fish and crustaceans store astaxanthin from food in their body tissues without changing its chemical structure [13], while lutein can be converted to astaxanthin and stored in its original form. Male rainbow fish are thought to be able to convert lutein into astaxanthin, while females retain it in its original form.



a. Fin of male fish fed control diet

b. The fin of female fish fed the control diet

c. Fin of male fish fed astaxanthin diet

d. The fin of female fish fed astaxanthin diet

e. Fin of male fish fed lutein diet

f. The fin of female fish fed lutein diet

Figure 4. The results of microscopy observations on the fins of rainbow ajamaru fish fed with different feeds

Overall, the results showed that the supplementation of astaxanthin and lutein increased the levels of red carotenoid pigments in male fish, while the supplementation of lutein was able to optimize the levels of yellow pigments in female fish.

4 Conclusion

The supplementation of astaxanthin and lutein in the diet increased the levels of carotenoid pigments in the fins and scales of the Ajamaru rainbowfish, with the highest values in the fins and skin of male fish being 202.90 and 8.53 ppm, respectively. The effect of astaxanthin and lutein supplementation on color can be observed microscopically in the tissues of scales and fins. The use of astaxanthin and lutein must be combined for the color of the Ajamaru rainbowfish to optimize the color of male and female fish.

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