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Biochemical Study on the Carotenoids in the Anemonefish, *Amphiprion* spp.

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Abstract

The carotenoid compositions of four species of wild anemonefish, *Amphiprion ocellaris, A. biacleatus, A. frenatus* and *A. clarkii*, were analyzed. All-*trans*-zeaxanthin was found to be a dominant pigment followed by *cis*-isomers of zeaxanthin in all four species of anemonefish. Astaxanthin was also isolated as a major carotenoid in these species except *A. clarkii*, in which no astaxanthin was detected. Astaxanthin, however, was isolated from the eggs of *A. clarkii*.

A feeding experiment was conducted using *A. ocellaris*. It was found that *A. ocellaris* was able to incorporate the dietary zeaxanthin and astaxanthin into the integuments. Both astaxanthin and zeaxanthin were effective for the pigmentation of *A. ocellaris*. However, astaxanthin gave a more desirable coloration for *A. ocellaris* than zeaxanthin.

The anemonefish, *Amphiprion* spp., are well known for their living in association with certain sea anemones. Because of their beautiful reddish-orange color they are a popular aquarium tropical fish and they are cultured in home and hatcheries. These fish, however, tend to lose red pigmentation on prolonged culture because of the lack of proper carotenoids in the diets.

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A number of papers on the carotenoid composition of red marine fish have been published[1-3]. According to these authors, astaxanthin is the carotenoid responsible for red pigmentation in fish and lutein and tunaxanthin are responsible for yellow color. Zeaxanthin has often been isolated from various fish, but it is usually a minor pigment.

It is generally assumed that carotenoids cannot be synthesized *de novo* by animals, but many fish have the metabolic capacity to modify dietary carotenoids. The pigments isolated from fish are either originated from the diet or are the result of a transformation of dietary carotenoids. The addition of proper carotenoids into the diet is required to improve and to maintain the desirable color in fish.

In the present study, the carotenoid compositions of four species of wild anemonefish, *Amphiprion* spp., were analyzed. The effects of the dietary zeaxanthin and astaxanthin for the pigmentation of *A. ocellaris* were investigated.

Materials and Methods

Anemonefish

Four species of wild anemonefish, *Amphiprion ocellaris, A. biacleatus, A. frenatus* and *A. clarkii*, were provided by Instant Ocean Hatcheries, Inc., Dade City, Florida, U.S.A.. These fish had been captured earlier at the Phillipine Islands.

A. ocellaris (0.4 g in average weight) used for the feeding experiment were also provided by Instant Ocean Hatcheries, Inc..

Feeding Experiment

One hundred twenty *A. ocellaris* were divided into three groups (Group 1−3). Fourty fish were maintained in each 50 liter fish tank. The following diets were fed to fish for 30 days.

Group 1 : basal diet (Rangen Salmon Starter, Ziegler Bros Feed Mills, Inc., Gardner, Pennsylvania, U.S.A.) fixed with 7% gelatin.

Group 2 : basal diet supplemented with 8mg/100 g all-*tras*-zeaxanthin

Group 3 : basal diet supplemented with 17mg/100 g astaxanthin

Analysis of Carotenoids

The extraction and saponification of carotenoids were performed according to Tanaka *et al*[4]. The crude carotenoids were first saponified and separated on MgO : Hyflo Super Cel = 1 : 2 (MgO) column with acetone in petroleum ether (PE). Zeaxanthin, astaxanthin and minor carotenoids fractions were detected. The zeaxanthin and astaxanthin fractions were re-chromatographed on $CaCO_3$ and sucrose columns for further purification. The minor pigments were further separated and purified by the thin layer chromatography (TLC) using silicagel-G plate.

The carotenoids in the eggs of *A. clarkii* were analyzed by the above mentioned methods without saponification.

Identification of Carotenoids

Carotenoids were identified in the following manner ; behavior and color of the chromatograms on column and TLC plate, absorption spectra in various solvents, chemical tests such as acetylation, methylation, reduction, epoxide tests and iodine-catalyzed photo-isomerization and co-chromatography with authentic standards.

Chemicals

Astaxanthin and all-*trans*-zeaxanthin were provided by F. Hoffmann-La Roche & Co., Basel, Switzerland.

Cis-isomers of zeaxanthin were prepared from all-*trans*-zeaxanthin by iodine-catalyzed photo-isomerization according to Zechmeister[5] and Hertzberg *et al.*[6]. The *cis*-isomers were purified on a $CaCO_3$ column developed with a benzene-hexane-acetone mixture (10 : 4 : 1).

Results and Discussion

Carotenoid Composition of Wild Anemonefish

The carotenoid content and a relative abundance in the integuments of four species of wild anemonefish, *A. ocellaris*, *A. biacleatus*, *A. frenatus* and *A. clarkii*, are shown in Table 1.. Zeaxanthin including *cis*-isomers (*cis*-A and *cis*-B) of zeaxanthin was isolated as a dominant pigment in all four species of anemonefish. As shown in Table 1., over 70 % of the total carotenoids was in some form of zeaxanthin. The

Table 1. Amounts and relative abundances of the carotenoids in four species of *Amphiprion*.

	A. ocellaris		*A. biacleatus*		*A. frenatus*		*A. clarkii*		*A. clarkii* (egg)	
Fish color	Reddish orange		Deep reddish orange		Reddish orange		Yellowish orange		Reddish orange	
Carotenoids	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)	(μg/g)	(%)
β-Carotene	t	t	t	t	t	t	t	t	–	–
β-Cryptoxanthin	t	t	t	t	t	t	t	t	t	t
Tunaxanthin	t	t	t	t	t	t	0.64	3.0	–	–
Lutein	6.21	6.4	9.57	6.0	4.26	7.4	2.19	10.3	t	t
All-*trans*-Zeaxanthin	53.16	54.8	88.25	55.3	32.33	56.2	11.53	54.2	167.13	78.8
9-*cis*-Zeaxanthin	11.64	12.0	20.91	13.1	6.50	11.3	4.15	19.5	t	t
13-*cis*-Zeaxanthin	6.69	6.9	11.81	7.4	3.22	5.6	2.77	13.0	t	t
Astaxanthin	19.30	19.9	29.04	18.2	11.22	19.5	–	–	44.96	21.2
Total carotenoid	97.00	100.0	159.04	100.0	57.50	100.0	21.23	100.0	212.00	100.0

content of *cis*-A and *cis*-B zeaxanthin was 20% and 10% of the total zeaxanthin content, respectively. Astaxanthin was the second highest carotenoid in content (20% of total carotenoid) in three species of anemonefish (*A. ocellaris*, *A. biacleatus* and *A. frenatus*), but it was not detected in the integument of *A. clarkii*. It was observed that the color of the previous three species was more reddish than that of *A. clarkii*. A small amount of β-carotene, β-cryptoxanthin, lutein and tunaxanthin was found in the integuments of all four species of anemonefish.

The carotenoid composition of the eggs of *A. clarkii* are also shown in Table 1.. All-*trans*-zeaxanthin (80% of total pigment) and astaxanthin (20% of total pigment) were found as the major carotenoids in the eggs. Only trace amount of the *cis*-isomers of zeaxanthin was detected. The carotenoid composition of the integument and the eggs of *A. clarkii* were quite different. As shown in Table 1., astaxanthin was found in the eggs but not in the integument. *Cis*-zeaxanthin was found in the integument at the level of 30% of total zeaxanthin but only a trace amount of *cis*-zeaxanthin was detected in the eggs.

The carotenoid compositions found in four species of wild anemonefish, *Amphiprion* spp. were rather unique. Over 70% of the total carotenoid in all four species was in some form of zeaxanthin. Astaxanthin is an important carotenoid for red color tone in *Amphiprion* spp. but zeaxanthin seemed to be the most important carotenoid for their characteristic orange-red coloration based on the carotenoid distribution. Other red colored fish have not been found to contain as an high amount of zeaxanthin as found in *Amphiprion* spp..

The carotenoid content of the organs were low and thus the carotenoid analysis was difficult because of the high level of lipids. From this experiment, it is difficult to suggest the origin of zeaxanthin and astaxanthin. They could be directly accumulated in the skin from the food or metabolized from other dietary carotenoids. The wild *Amphiprion* spp. are known to be omnivorous feeders subsisting primarilly on benthic algae and planktonic crustaceans (copepods)[7]. It seems likely that zeaxanthin in the integuments could be derived from the algae and astaxanthin from the crustaceans.

Two yellow zeaxanthin-like pigments (pigment A and B) were isolated from the fraction close to the chromatographic positions of all-*trans*-zeaxanthin. These fractions were adsorbed above the corresponding all-*trans*-zeaxanthin on a $CaCO_3$ column developed with a benzene-hexane-acetone mixture (10 : 4 : 1). Acetylation test of both pigments showed that these pigments contained two hydroxyl groups. While, methylation, reduction and epoxide tests were negative.

Absorption spectra in acetone of pigment A and B were at 345, (425), 449 and 476 nm and 343, (425), 448 and 474 nm, respectively. While all-*trans*-zeaxanthin showed at (428), 453 and 478 nm. *Cis*-peaks appeared at 345 nm and 343 nm, for pigment A and B, respectively. A hyperchromic shift of 1–3 nm observed after the photo-isomer-

ization of pigment A and B indicated the irreversibility of *cis-trans* conformation. Co-chromatography of pigment A and B with authentic *cis*-zeaxanthin identified the pigment A and B were *cis*-isomers of zeaxanthin (*cis*-A and *cis*-B). The 9-*cis*- and 13-*cis*-zeaxanthin are most commonly formed after the iodine-catalyzed photo-isomerization[5,6]. These *cis*-isomers were identified based on spectral characteristics (λ max shifts, *cis*-peak intensity, IR spectrum[5], ^{13}C-NMR[8] and CD spectrum[6]). The chromatographic positions on column, spectral characteristics including absorption spectra, *cis*-peak intensity and max shifts of *cis*-A and *cis*-B agreed with literature values[5,6] of 9-*cis*- and 13-*cis*-zeaxanthin, respectively. It was suggested that *cis*-A was 9-*cis*-zeaxanthin and *cis*-B was 13-*cis*-zeaxanthin. The more detailed analyses will be required for the further conformation.

Cis-isomers of zeaxanthin were found at a level of 30 % of the total zeaxanthin in the integuments of the wild anemonefish. *Cis*-forms of carotenoids have not been isolated in aquatic animals with exception of the sea sponges[9]. It is well known that *cis-trans* isomerization of carotenoids occurs during isolation procedures promoted by heat, light, acid, active surfaces etc. The *cis*-isomers of zeaxanthin isolated from the integuments of the anemonefish were not the results of isomerization from *trans*-zeaxanthin during isolation procedures. Because the absorption spectrum of the crude extract from the integuments of *A. clarkii* showed that *cis*-peak had already been found in the near ultra violet region before saponification. In addition, only *trans*-zeaxanthin was isolated from the eggs of *A. clarkii* although a trace amount of *cis*-zeaxanthin was detected in the eggs. If the *cis*-isomers of zeaxanthin isolated from the integuments were artificially formed from all-*trans*-zeaxanthin during analysis of carotenoids, they should be isolated from the eggs at the same ratio as found in the integuments. At this point, it is difficult to determine whether the formation of *cis*-zeaxanthin is enzymatic metabolism or is merely formed as a result of strong sunlight destruction in the tropical regions.

Feeding Experiment

At the end of 30 day's feeding experiment, *A. ocellaris* showed a light yellow-orange to pinkish orange color. The fish fed with astaxanthin showed a pinkish orange color. The fish fed with zeaxanthin were light orange and the control fish were yellowish orange color. The carotenoid composition and a relative abundance in *A. ocellaris* are shown in Table 2.. Both test groups (Group 2 and Group 3) contained much higher level of carotenoids than that of the control group. In the group 3, astaxanthin, zeaxanthin and β-carotene were found to be the major carotenoids and a small amount of echinenone, canthaxanthin and β-cryptoxanthin were detected. It was also found that the level of zeaxanthin in group 3 was 2 times higher than that of the control group. In the group 2, zeaxanthin was found as the major pigment followed by β-carotene and lutein. The highest level of zeaxanthin was isolated from the group 2.

Table 2. Carotenoid composition of *Amphiprion ocellaris* fed with the diets containing zeaxanthin and astaxanthin for 30 days

	Basal diet[a]		Control		Zeaxanthin		Astaxanthin	
Fish color			Light yellow		Light orange		Pinkish orange	
Carotenoids	($\mu g/g$)	(%)	($\mu g/g$)	(%)	($\mu g/g$)	(%)	($\mu g/g$)	(%)
β-Carotene	4.78	55.3	8.94	50.5	7.97	20.4	12.98	26.4
Echinenone	–	–	–	–	–	–	2.36	4.8
Canthaxanthin	–	–	–	–	–	–	0.54	1.1
β-Cryptoxanthin	–	–	–	–	t	t	0.74	1.5
Tunaxanthin	–	–	–	–	–	–	0.69	1.4
Lutein	1.71	19.8	2.98	16.8	5.35	13.7	2.26	4.6
Zeaxanthin[b]	2.15	24.9	5.79	32.7	25.74	65.9	11.16	22.7
Astaxanthin	–	–	–	–	–	–	17.85	36.3
Unknown	–	–	–	–	–	–	0.59	1.2
Total carotenoid	8.65	100.0	17.71	100.0	39.06	100.0	49.17	100.0

[a] Rangen Salmon Starter (Ziegler Bros Inc) fixed with gelatin.

[b] 9-*cis* and 13-*cis* were present at about 20% and 10% of the total zeaxanthin content in all fishes, while trace amount of *cis*-isomers were present in the basal diet.

This evidence is a good indication that *A. ocellaris* can incorporate dietary astaxanthin and zeaxanthin. In the control group β-carotene and lutein were isolated along with a lower amount of zeaxanthin compared to the group 2 and 3. Astaxanthin, however, was not detected in the group 2 and the control.

These differences of carotenoid composition directly reflected their visual color appearance of skin. A pinkish orange color of group 3 may be due to astaxanthin and zeaxanthin, and a orange color of group 2 are because of zeaxanthin. The control fish showed more yellowish color than the group 2 and 3. This is obviously due to the lack of astaxanthin and a much less amount of zeaxanthin. These results clearly showed that astaxanthin and zeaxanthin were two most important carotenoids for the pigmentation of *A. ocellaris*.

It is assumed that astaxanthin improves the coloration of *A. ocellaris* and gives a more natural color to this species. Zeaxanthin is required for the orange coloration. In other species of anemonefish, *A. biacleatus* and *A. frenatus*, it is assumed that astaxanthin would be responsible for the red pigmentation and zeaxanthin is for orange color according to their carotenoid composition which was similar to *A. ocellaris*.

An interesting result was obtained from *A. ocellaris* fed with the astaxanthin diet. In this group, echinenone, canthaxanthin, β-cryptoxanthin and tunaxanthin were isolated. These pigments were not isolated from the control and the zeaxanthin fed groups and the diet. It was also found that the level of zeaxanthin in group 3 was the 2 times higher than that of the control group. These results suggested that two possible metabolic pathways of astaxanthin might be present in *A. ocellaris*. One

would be the conversion of astaxanthin into echinenone through canthaxanthin and another would be the conversion of astaxanthin into zeaxanthin.

The reactions of carotenoid metabolism in animals are essentially oxidative. However, the pathway of reductive metabolism in aquatic animals have recently been discovered. Kitahara[10] first reported the conversion of astaxanthin into zeaxanthin in chum salmon. Since then, the reductive pathways of the carotenoids in aquatic animals, such as rainbow trout[11,12] and yellowtail[13], have been reported.

The significantly increased zeaxanthin level in the group 3 fish compared to that of the control group may suggest that *A. ocellaris* are able to convert astaxanthin into zeaxanthin as found in the rainbow trout[11,12], chum salmon[10] and yellowtail[13] although the intermediate carotenoids such as 3,4,3'-triol-β, β-carotene were not isolated. The conversion of astaxanthin to echinenone was not clear in this experiment because of relatively low levels of echinenone and canthaxanthin were found. A more detailed experiment will be required to confirm the conversion of astaxanthin to zeaxanthin or echinenone.

In red colored marine fish, astaxanthin is the most important carotenoid reproducing a red color in the integument of the fish. Likewise, in *A. ocellaris* astaxanthin was an important carotenoid for the improvement of the fish color tone, and zeaxanthin was also the important carotenoid for the characteristic orange coloration in anemonefish. Zeaxanthin is not a rare pigment in animals and plants, but it usually occurs as a minor pigment. The wild anemonefish contained a quite high level of zeaxanthin. The source of zeaxanthin is unknown but is reasonable to suggest that zeaxanthin would be from the diet directly or metabolized from other dietary carotenoids, especially from astaxanthin. Normally, red colored marine fish can selectively deposit the carotenoids from the food without modification. Although astaxanthin could be reduced to zeaxanthin in certain species of anemonefish such as *A. ocellaris*, it seems that the most of the carotenoids accumulated in the integuments is selectively derived from the fish's natural food without modification.

In this study, the carotenoid compositions of four species of *Amphiprion* spp. were analyzed and the possible metabolic pathways were suggested. The results of carotenoid analysis and the feeding experiment suggested that the two most important carotenoids to maintain and to improve the characteristic natural color in the anemonefish are astaxanthin which is responsible for the red coloration and zeaxanthin which is responsible for the orange coloration. The diets should contain these carotenoids at the proper level to improve the color tone of the cultured fish.

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