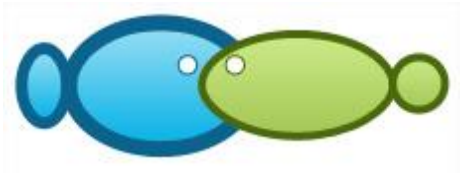

Natural carotenoids extracted from red bell pepper for enhancement of growth and coloration of false clownfish, *Amphiprion ocellaris*

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Abstract. This study aimed to evaluate the effects of natural carotenoids extracted from red bell pepper (*Capsicum annuum*) on the growth and body pigmentation of the false clownfish *Amphiprion ocellaris*. The carotenoid diets supplied to the clownfish were set up with serial concentrations of 0–1.5 g kg^{-1} feed. Our data revealed that the carotenoid feed significantly affected the false clownfish's growth performance and skin pigmentation. After 75 days on the 0.3–0.9 g kg^{-1} concentration carotenoid diet, a high redness (a^*) value was observed in the range of 13.49–18.59 (Minolta, CR400), while the control group demonstrated an a^* value of only 7.95±0.23. The highest carotenoid level (121.95±6.44 μg g^{-1} tissue, UV-vis spectrophotometer) was also found in the fish skin of the group with a dietary carotenoid concentration of 1.5 g kg^{-1} feed. Significant differences in fish growth were also observed within the high dietary carotenoid groups compared with the control group. It is suggested that the optimal carotenoid level (0.9 g kg^{-1} feed) from red bell peppers helps improve body pigmentation and the growth performance of the false clownfish, and these plant-derived carotenoids have a potential application in aquaculture.
Key Words: aquaculture, *Capsicum annuum*, marine ornamental fish, natural pigments.

Introduction. In recent years, ornamental fish farming has become a valuable industry, and the color of fish skin is used as a primary indicator of fish quality and market prices (Sathyaruban et al 2021; Singh et al 2021). In nature, fish are unable to biosynthesize carotenoids, but they can accumulate these pigments in their bodies by consuming phytoplankton and some microalgae (Gupta et al 2007; Boonyapakdee et al 2015; Das & Biswas 2016). Whereas, when fish are cultured in high-density, in captive conditions without the supplementation of dietary carotenoids, it can lead to faded pigmentation, which decreases the commercial value of the fish and directly affects consumer acceptance or rejection (Wassef et al 2010; Maiti et al 2017).

Amphiprion ocellaris belongs to the family Pomacentridae and is globally known as one of the most popular species due to its vibrant color, value and high demand in the marine ornamental markets. It is also an ideal research species due to its regular spawning and ease of maintenance under laboratory conditions (Ebeneezar et al 2020).

Previous studies found that natural and synthetic pigments can be applied to improve the appearance of several fish species (Gouveia & Rema 2005; Pham et al 2014; Yi et al 2018; Zhang et al 2020). The problem, however, is that synthetic components come at a high cost to farmers, and there are concerns about the safety of using artificial colorants. This has led researchers to investigate more natural and cost-effective sources of carotenoids utilized in agriculture practices (Gupta et al 2007; García-Chavarría & Lara-Flores 2013; Maiti et al 2017). Plant-based natural carotenoids (fruits, flowers, roots, vegetables, and others) are reported to be a source of mixed carotenoids (Singh et al 2021). The consumption of carotenoid-rich food not only improves the body pigmentation

of fish, but has also been associated with numerous health benefits, such as anti-oxidation, enhanced immunity, stress resistance, reproduction, and resistance to bacterial and fungal diseases (García-Chavarría & Lara-Flores 2013; García-Romero et al 2014; Yi et al 2018).

Red bell pepper (*Capsicum annum*) belongs to the family *Solanaceae*. The red pigments are known as keto-carotenoids: capsanthin, capsorubin, and cryptoxanthin are unique to the *Capsicum* species and known for their primary carotenoid content, while other xanthophylls (yellow-orange color) are also found, such as violaxanthin, zeaxanthin, β-cryptoxanthin, and β-carotene (Wassef et al 2010; Rhim & Hong 2011; Maniat et al 2014).

In terms of natural carotenoids, almost all natural pigments added to the feed are currently applied in raw form (raw materials containing carotenoids are directly added to the feed). This approach has some drawbacks, such as low carotenoid concentrations in materials, requiring high quantities, an effect on nutritional balance when adding a large amount of pigment-containing materials to the diet, and low digestion due to pigments in complex bonds or in the cells of materials (Tran et al 2022). For the above reasons, our study was conducted to evaluate the effect of semi-purified carotenoids extracted from red bell pepper on the skin pigmentation and growth performance of false clownfish. The findings of this study may affirm the need to investigate various plants and agricultural by-products to provide a cheap, natural, and safe option for aquaculture farmers to increase the value of their stocks.

Material and Method

Ethics statement. The protocols were conducted following the National Regulations for the Use of Animals in Research in Vietnam (The Law of Animal Husbandry of Vietnam, 2018), and The Government Decree 32/2006/ND-CP on Management of Endangered, Precious, and Rare Species of Wild Plants and Animals, experiments on marine fishes are exempt from ethical approval requirements). During the experimental period (rearing, handling and sampling), fish were reared in optimal conditions, and efforts were made to minimize stress.

Preparation of carotenoids. The red bell peppers were collected from a farm (Dien Lac Commune, Dien Khanh District, Khanh Hoa Province, Vietnam). The fresh material (100 g) was ground in a grinder using 300 mL of ethanol 96% as a solvent. The sample was extracted using a microwave oven (Sharp microwave 900W, frequency 2450 MHz, Japan) as described by Dang et al (2018). The extract was filtered using a cloth, and the process was repeated three times until the filtrate became colorless. The pooled extract was collected, and the filtrates were concentrated using a rotary evaporator (IKA RV 10 control V, Germany). Thereafter, 50 mL of sunflower oil was added to the extracts to dissolve the carotenoids. Next, the carotenoid-enriched oil was washed with hot water (temperature of 80°C) to remove any residues. Finally, the carotenoid oil was dried using anhydrous sodium sulfate and measured using a UV-visible spectrophotometer to determine the level of carotenoids in the sample.

Experimental diets and preparation. The basal diet (with 55% crude protein and 12% lipid) was formulated according to marine finfish feed requirements (Liu et al 2014; Nankervis et al 2022). Serial carotenoid levels were added in the following concentrations: the control diet 0 g kg^{-1} (D0), 0.3 g kg^{-1} (D0.3), 0.6 g kg^{-1} (D0.6), 0.9 g kg^{-1} (D0.9), 1.2 g kg^{-1} (D1.2), and 1.5 g kg^{-1} (D1.5) (Table 1). Our procedure followed the method outlined by Ebeneezar et al (2020) with minor modifications. The ingredients for feed (except for carotenoid oil and vitamin mix) were weighed as per formula and mixed homogenously in a mixer. After mixing and adding water, the dough for feed was cooked in a pressure cooker for 20 min. After cooking and cooling, the dough with added carotenoid oil and vitamin mix was extruded through an 800 µm mesh. The feed was oven-dried at 60°C for 8 h, then crumbled and sieved to form pellets. The feeds were packed in air-tight plastic containers and refrigerated until use.

Table 1

Formulation and proximate chemical composition of the experimental diets (g kg^{-1})

Ingredient	*D0*	*D0.3*	*D0.6*	*D0.9*	*D1.2*	*D1.5*
Fishmeal (Peru) (g)	470	470	470	470	470	470
Fishmeal (Vietnam) (g)	180	180	180	180	180	180
Squid meal (g)	170	170	170	170	170	170
Corn gluten meal (g)	115.9	115.6	115.3	115.0	114.7	114.4
Soybean oil (g)	45.6	45.6	45.6	45.6	45.6	45.6
Vitamin premix[1] (g)	12.8	12.8	12.8	12.8	12.8	12.8
Lysine (g)	0.5	0.5	0.5	0.5	0.5	0.5
Methionine (g)	0.2	0.2	0.2	0.2	0.2	0.2
Mineral premix[2] (g)	5	5	5	5	5	5
Carotenoids supplement (g kg^{-1})	0	0.3	0.6	0.9	1.2	1.5
Chemical and proximate composition (%)						
Crude protein	55.03	54.98	55.07	55.16	55.17	55.19
Crude lipid	12.01	12.09	11.97	12.13	12.18	12.21
Ash	11.16	11.21	11.23	11.19	11.17	11.22
Moisture	10.04	10.08	9.92	10.13	10.15	10.11
Carotenoids (g kg^{-1})	0.03	0.33	0.64	0.95	1.24	1.53

Note: [1] vitamin premix (mg kg^{-1} diet): vitamin A, 1000000 IU; vitamin D3, 300000 IU; vitamin C monophosphate, 10000 mg; pantothenic acid, 2500 mg; vitamin E, 2000 mg; vitamin B3, 2000 mg; vitamin K3, 500 mg; vitamin B1, 500 mg; vitamin B6, 500 mg; vitamin B2, 320 mg; folic acid, 200 mg; biotin, 20 mg; vitamin B12, 5 mg; inositol, 10 mg; choline chloride, 5 mg. Provimi Vietnam Co. Ltd., Bien Hoa city, Dong Nai province, Vietnam; [2] mineral premix (mg kg^{-1} diet): Zn (ZnO), 4750 mg; Mn ($MnSO_4.H_2O$), 1900 mg; Mg (MgO), 1050 mg; Co ($CoCO_3$), 47.5 mg; Se (Na_2SeO_2), 47.5 mg; I ($Ca(IO_2)_2.H_2O$), 19 mg; P ($CaHPO_4.2H_2O$), 0.7%; Ca ($CaHPO_4.2H_2O$), 0.8%; moisture, 10%; ash, 2%; ethoxyquin, 240 mg; carrier (dextrose), 86%, Provimi Vietnam Co. Ltd., Bien Hoa city, Dong Nai province, Vietnam.

Fish rearing conditions. False clownfish were bred and reared at the Vinh Hoa Marine Ornamental Fish Hatchery, Institute of Aquaculture, Nha Trang University, Vietnam. Before the experiment, fish were acclimatized to laboratory conditions for two weeks and fed a commercial diet (INVE, Ltd., Thailand). 18 rectangular glass tanks (L×W×H=55×35×38 cm) were filled with 65 L of water, and 15 fish were placed into each tank. Continuous aeration was set up in each tank through an air system to maintain oxygen saturation. Filtered seawater was constantly circulated in the tanks with a regulated flow rate of 1.5 L per minute per tank. The water was changed weekly, with one-third of the water being refreshed. The fish were fed with carotenoid diets of 0–1.5 g kg^{-1} (each experiment was triplicated), four times daily at 7:00, 10:00, 13:00 and 16:00. The quality of the water was maintained at the following parameters: temperature, 28.5±1.53°C; salinity, 33.2±0.95‰; pH, 7.94±0.15; oxygen, 5.6±0.14 mg L^{-1}; unionized ammonia nitrogen, 0.04±0.01 mg L^{-1}. The feeding trial was subjected to a natural light and dark cycle for the entire 75-day experimental period.

Growth performance and physiological status. At the end of the experiment, all fish from each tank were starved for 24 h and anesthetized with 0.05% ethylene glycol monophenyl ether (Merck KGaA, Darmstadt, Germany) before measuring growth performance, physiological status and skin color. The growth performance and physiological status of the groups were measured, including the following: length gain (LG), weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), feed efficiency ratio (FER), protein efficiency ratio (PER), condition factor (CF), and survival rates (SR).

Length gain, LG (%) = 100 × (final body length, cm - initial body length, cm)/initial body weight (cm)

Weight gain, WG (%) = 100 × (final body weight, g - initial body weight,g)/initial body weight (g)

Specific growth rate, SGR_L (%) = 100 × [ln(final length, cm) – ln(initial length, cm)]/days of the experiment (75)

Specific growth rate, SGR_W (%) = 100 × [ln(final weight, g) – ln(initial weight, g)]/days of the experiment (75)

Condition factor, CF (g/cm^3) = 100 × (body weight, g)/(total length, cm)^3

Feed intake, FI (g/fish) = total consumed feed (g) / number of fish

Feed conversion ratio, FCR = feed intake (g, dry weight)/[final body weight (g) – initial body weight (g)]

Feed efficiency ratio, FER (%) = 100 × [final body weight (g) – initial body weight (g)]/feed intake (g)

Protein efficiency ratio, PER (%) = 100 × [final body weight (g) – initial body weight (g)]/protein intake (g).

Survival rate, SR (%) = 100 × (final number of fish/initial number of fish)

Color measurement. Fish pigmentation was measured on both sides of the fish body at the position between the soft dorsal fin and anal fin adjacent to the white middle band of the fish. Triplicate measurements were taken at each position using a chroma meter CR-400 (Konica Minolta, Osaka, Japan). Assessment of skin pigmentation was performed by reflectance spectroscopy. The color parameters were L^* values for lightness ranging from 0–100 for black to white, a^* values for red/green, and b^* values for yellow/blue. For example, an L^* value of 100 indicates absolute brightness, whereas a value of 0 designates absolute darkness. The ($^+$) higher positive 'a^*' and 'b^*' values show a higher level of redness and yellow-orangeness, while ($^-$) negative values of 'a^*' and 'b^*' express green and blue, respectively.

Chemical analysis. The proximate compositions of the experimental diets were analyzed according to the standard methods outlined in AOAC (2005). The samples were dried to a constant weight at 105°C in an oven to determine moisture content. Crude protein (Nx6.25) was analyzed using the Kjeldahl method. Crude lipid was determined by ether extraction using the Soxhlet system, and ash content was measured after combustion at 550°C for 24 h (Table 1).

Carotenoids from red bell peppers and fish skin were isolated and analyzed on a thin layer chromatography (TLC) plate (20x20 cm, Kieselgel 60F254, 0.25 mm, Merck, Castle Hill, NSW, Australia) as the stationary phase. A mixture of hexane, petroleum ether, chloroform and acetone with different ratios was used as the mobile phase.

Total carotenoid content in the feed, skin, muscle, and the whole body of the fish was analyzed using a UV-visible spectrophotometer following the method described by Ramamoorthy et al (2010) and García-Romero et al (2014) with minor modifications. Samples of skin (0.25 g) were collected from both sides of the fish, while muscle (0.25 g), whole body (1 g), and feed (1 g) samples were also prepared. The samples were ground in acetone (20 mL) containing 1.5 g of anhydrous sodium sulfate with a homogenizer (model T10, Ultra-turrax ®, IKA, Germany). The samples were then filtered using filter paper, and the process was repeated three times until the filtrate became colorless. The filtrates were centrifuged at 10000 rpm at a temperature of 4°C for 15 min. Lastly, absorption was measured at 485 nm using a spectrophotometer (Biochrom Ltd, Cambridge, England). The results were expressed as micrograms per gram (μg g^{-1}) and calculated using the following equation.

Total carotenoid content (μg g^{-1}) = $AxDxVx10^4/WxE^{1\%}_{1cm}$

Where: A is the absorbance; V is the total volume of the extract (mL); D is the dilution ratio; W is the weight of the sample (g); and an extinction coefficient (E) of 2100.

Statistical analyses. All experiments were triplicated. The data were expressed as mean ± standard error (n=3). A one-way ANOVA and Duncan's post hoc test were used (SPSS Statistical Software, Version 22.0) to analyze the differences between the samples. Differences between the mean levels of the carotenoids, color expression and growth parameters of fish in different experiments were considered statistically significant if they had a p-value of <0.05.

Results and Discussion

Carotenoids from red bell pepper and fish. The carotenoid yield from the red bell pepper at 4.03±0.15 mg g^{-1} wet weight was observed with a ratio of ethanol 96% to material (3/1; v/w).

Thin-layer chromatography (TLC) is useful in the analysis and detection of components in plants and animals. Several compounds were found in the extracts of red bell pepper and fish. Totally, 11 components (three yellow bands: C1, C5, C10; five red bands: C2, C6, C7, C8, C11; one pink band: C4, and two orange bands: C3, C9) were seen in the extraction of red bell pepper, while five constituents (F1–F5) were found in the fish sample (Figure 1). The mixture of hexane and acetone (3:1) as a mobile phase is suitable for separating components in the red bell pepper. Yellow bands (C1) [retention factor (R_f)=0.96] may be *β*-carotene. The following strong red band (C2) could be capsanthin (R_f=0.82). For the carotenoid profile of fish, it is suggested that the strong yellow band (F1, R_f=0.95) was *β*-carotene. The three other light carotenoids (red bands: F2, F3, F4) were astaxanthin, while the yellow band (F5, R_f=0.13) could be lutein or zeaxanthin.

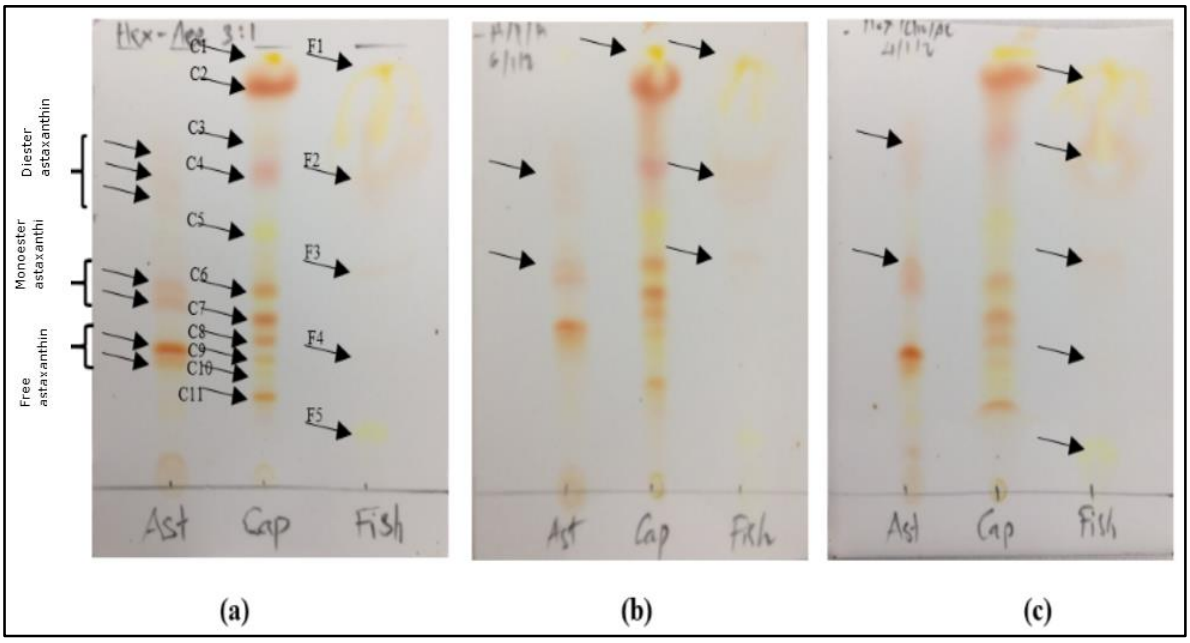

Figure 1. Thin layer chromatogram of the standard astaxanthin (Ast.), *Capsicum annum* (Cap.) and fish skin extract (Fish) with the different mobile phases: (a) hexane/acetone (3/1); (b) hexane/petroleum ether/acetone (6/1/2); (c) hexane/chloroform/acetone (4/1/2).

Growth parameters and physiological status. The results of the measured growth and physiological parameters at the start of the experiment and as a result of feeding for 75 days are presented in Table 2. The average initial body weight and total length of fish were

0.72±0.02 g, and 3.4±0.04 cm, respectively, and the diets (with and without carotenoids) were equally accepted by the fish. At the end of the experiment, these values were 1.22-1.46 g and 4.02-4.31 cm, respectively. We found significant differences ($p<0.05$) in both the weight and length of fish subjected to carotenoid diets at concentrations of ≥0.9 g kg^{-1} compared with the control diet. Similarly, the differences in the values of LG, WG, SGR_L, SGR_W, FI, FCR, FER, and PER with high carotenoid treatments (≥0.9 g kg^{-1}) were statistically significant ($p<0.05$; Table 2). Conversely, there were no significant differences observed in the values of SR, and CF among the high carotenoid groups and the control sample ($p>0.05$).

Effect of carotenoid diet on skin pigmentation. Data on color parameters (L^*, a^*, b^*) for pigmentation in the false clownfish fed the carotenoid diets for 75 days are reported in Table 3 and Figure 2. The results indicate that supplementing carotenoids from red bell peppers led to a significant improvement in the red pigmentation (the values of a^*) of the false clownfish ($p<0.05$). Remarkably, there was a statistically significant difference in the redness between the control and all carotenoid groups ($p<0.05$). The carotenoid-enriched feed with a concentration of 0.9 g kg^{-1} resulted in the highest a* value (redness), which was measured at 18.59±0.48. Notably, even for the lowest tested carotenoid concentration of 0.3 g kg^{-1}, the a* value was 1.7 times higher than that observed in the control sample. Red pigmentation in fish increased with the increase of supplemental carotenoids. However, no significant differences in red pigmentation of fish were found when supplemented carotenoid concentrations were higher than 0.9 g kg^{-1} feed, with a decreasing tendency. It is clear that carotenoids enhanced the redness of the fish skin over the 75-day experimental period, and the carotenoid diet of 0.9 g kg^{-1} feed was the best for improving the skin pigmentation. Conversely, the lightness (the values of L^*) remained stable (except for lightly lower values of L^* when carotenoids >0.9 g kg^{-1} were added), while the yellowness (the values of b^*) of fish increased and peaked in the treatment with carotenoids of 0.9 g kg^{-1} and decreased slightly at higher concentrations.

Analysis of total carotenoid content. Based on the above results, it is evident that the amount of carotenoids accumulated in the skin of fish groups fed with a carotenoid diet was improved in comparison to the control group. Additionally, a strong correlation exists between the red pigmentation (a* value) and the carotenoid content in fish skin (Tables 3 and 4). The highest carotenoid concentration, 121.95±6.44 µg g^{-1}, was found in the tissue of the fish fed a diet containing 1.5 g kg^{-1} of carotenoids, which was more than three times higher than that of the control group (37.43±5.3 µg g^{-1}). Additionally, the accumulated carotenoid content in fish skin was high (74.15±5.84 µg g^{-1}) even for the feed sample with the lowest carotenoids compared with the control sample. Furthermore, the statistically significant differences in total carotenoid content of fish skin were observed at all dietary carotenoid levels compared with the control sample ($p<0.05$). However, when the amount of carotenoids added to the feed was equal to or higher than 0.9 g kg^{-1}, the amount of carotenoids accumulated in the fish skin did not increase much, and there was no significant difference in the amount of carotenoids accumulated in the fish skin ($p>0.05$). Clearly, the carotenoids from red bell pepper added to the diet with the concentration of 0.9 g kg^{-1} were suitable for improving both red pigmentation and total carotenoid content of *A. ocellaris* skin.

Table 2

Growth parameters, somatic indices and survival of *Amphiprion ocellaris* fed diets containing different carotenoid levels

Parameters	*Dietary carotenoid levels (mg kg^{-1})*					
	D0	*D0.3*	*D0.6*	*D0.9*	*D1.2*	*D1.5*
L_1 (cm)	3.4±0.04	3.4±0.04	3.4±0.04	3.4±0.04	3.4±0.04	3.4±0.04
W_1 (g)	0.72±0.02	0.72±0.02	0.72±0.02	0.72±0.02	0.72±0.02	0.72±0.02
L_2 (cm)	4.02±0.04^{a}	4.05±0.02^{a}	4.07±0.06^{a}	4.26±0.03^{b}	4.21±0.02^{b}	4.31±0.06^{b}
W_2 (g)	1.22±0.02^{a}	1.23±0.01^{a}	1.27±0.03^{a}	1.44±0.03^{b}	1.41±0.05^{b}	1.46±0.06^{b}
LG (%)	18.1±1.18^{a}	19±0.61^{a}	19.73±1.78^{a}	25.47±0.86^{b}	23.77±0.71^{b}	26.90±1.92^{b}
WG (%)	69.43±2.57^{a}	71.47±2.16^{a}	76.27±4.42^{a}	100.4±4.49^{b}	95.37±6.51^{b}	103.30±8.68^{b}
SGR_L (%/d)	0.22±0.01^{a}	0.23±0.01^{a}	0.24±0.02^{ab}	0.3±0.01^{c}	0.28±0.01^{bc}	0.31±0.02^{c}
SGR_W (%/d)	0.7±0.02^{a}	0.72±0.02^{a}	0.76±0.03^{a}	0.93±0.03^{b}	0.89±0.04^{b}	0.94±0.06^{b}
CF (%)	1.88±0.28^{ab}	1.9±0.01^{ab}	1.9±0.04^{b}	1.88±0.02^{ab}	1.87±0.03^{ab}	1.81±0.01^{a}
FI (g/fish)	1.01±0.03^{a}	0.97±0.01^{a}	1.07±0.04^{a}	1.24±0.07^{b}	1.26±0.06^{b}	1.32±0.08^{b}
FCR	2.02±0.03^{d}	1.88±0.03^{bc}	1.96±0.04^{cd}	1.72±0.02^{a}	1.85±0.05^{bc}	1.79±0.05^{ab}
FER (%)	49.53±0.61^{a}	53.24±0.84^{bc}	51.19±0.93^{ab}	58.26±0.76^{d}	54.23±1.56^{bc}	56.02±1.55^{cd}
PER (%)	90.06±1.11^{a}	96.80±1.51^{bc}	93.08±1.68^{ab}	105.92±1.39^{d}	98.61±2.84^{bc}	101.86±2.82^{cd}
SR (%)	95.33± 2.33^{a}	95.33±2.33^{a}	97.67± 2.33^{a}	97.67±2.33^{a}	95.33± 2.33^{a}	97.67±2.33^{a}

Note: values represent means ± SE of three replicates; L_1 - initial total length; W_1 - initial body weight; L_2 - final total length; W_2 - final body weight; LG - percentage of length gain; WG - percentage of weight gain; specific SGR_L - growth rate in length; SGR_W - specific growth rate in weight; FCR - feed conversion ratio; FER - feed efficiency ratio; PER - protein efficiency ratio; CF - condition factor; FI - feed intake; SR - survival rate; different superscripts show significant differences ($p<0.05$).

Table 3
Skin pigmentation of *Amphiprion ocellaris* fed diets containing different carotenoid levels for 75 days

	Dietary carotenoid levels (mg kg^{-1})					
	D0	*D0.3*	*D0.6*	*D0.9*	*D1.2*	*D1.5*
L*	45.74±0.66^{c}	43.87±1.24^{bc}	45.67±0.57^{c}	44.07±0.43^{bc}	43.28±0.20^{ab}	41.32±0.29^{a}
a*	7.95±0.23^{a}	13.49±0.84^{b}	14.24±0.18^{b}	18.59±0.48^{d}	17.59±0.66^{cd}	16.63±0.26^{c}
b*	17.26±0.58^{a}	18.56±0.97^{ab}	21.07±0.87^{bcd}	22.27±0.34^{d}	21.81±0.66^{cd}	19.02±1.44^{abc}

Note: different superscripts show significant differences ($p<0.05$).

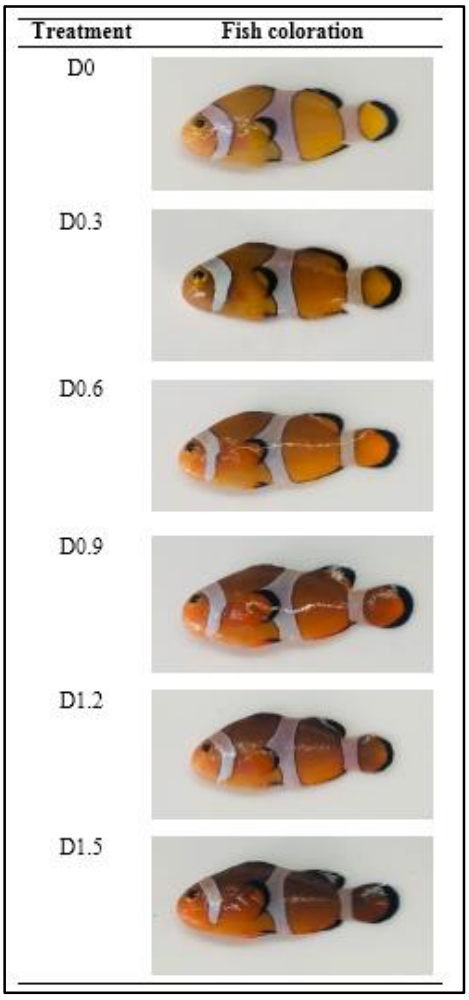

Figure 2. Results of *A. ocellaris* coloration fed diets containing different carotenoid levels for 75 days.

Similar trends were observed for the carotenoid content of the whole fish and muscles. The highest carotenoid values (30.75±2 μg g^{-1} for whole fish and 8.41±0.65 μg g^{-1} for muscle) were found at the dietary carotenoid level of 1.2 g kg^{-1} and 0.9 g kg^{-1}, while those for the control groups were of 14.34±1.39 μg g^{-1} and 2.27±0.64 μg g^{-1}, respectively. The statistical differences in total carotenoid content with all dietary carotenoid groups compared with the control groups were detected in both whole fish and muscles ($p<0.05$*;* Table 4).

It is reported that approximately 10 million tons of carotenoids are produced annually from biosynthetic organisms: high plants, algae, fungi, and bacteria (Schweiggert & Carle 2017). In red bell peppers, several main carotenoids were separated and identified. The coloration of the materials affects their carotenoid profiles. Capsanthin and capsorubin are the main carotenoids in red peppers, while *β*-carotene and lutein are abundant in orange, yellow and green peppers (Kim et al 2016). Other carotenoids such as violaxanthin,

zeaxanthin, cryptoxanthin, antheraxanthin, cucurbitaxanthin, capsanthone, neoxanthin, lycopene and some unidentified compounds can also be found (Wassef et al 2010; Arimboor et al 2015; Kim et al 2016). The carotenoids extracted from red pepper could be easily separated on silica gel plates using a mixture of petroleum ether–hexane–acetone, and a higher R_f value was seen for *β*-carotene in comparison to other carotenoids (Zeb & Murkovic 2010). According to Hernández-Ortega et al (2012), the yellow bands of three types of pepper with Rf=0.98 were *β*-carotene, while the red bands (Rf=0.91 and 0.34) were capsanthin and capsorubin, respectively. Carotenoid pigments can be deposited either directly within the chromatophore cells of fish and/or converted through cellular metabolism (Sathyaruban et al 2021). Fish can reduce as well as oxidize dietary carotenoids. The reduction and oxidation of carotenoids help in their transformation from one form to another. Deposition of carotenoids in different fish species occurs in different areas such as integuments, gonads and muscles (Wassef et al 2010; Singh et al 2021). The coloration of ornamental fish is influenced by several internal and external factors: chromatophores, species, genetics, developmental stages, and health status, as well as by nutritional supplementation, environmental factors, and social interaction (Wassef et al 2010; Sköld et al 2016; Sathyaruban et al 2021; McLean 2021; Luo et al 2021).

Table 4

Total carotenoid content (μg g^{-1}) in the whole body, skin and muscle of *Amphiprion ocellaris* fed diets containing different carotenoid levels for 75 days

	Dietary carotenoid levels (μg g^{-1})					
	D0	*D0.3*	*D0.6*	*D0.9*	*D1.2*	*D1.5*
Whole body	14.34 ±1.39^{a}	19.77 ±1.61^{b}	23.1 ±1.69^{b}	30.35 ±1.45^{c}	30.75 ±2^{c}	29.62 ±1.21^{c}
Skin	37.43 ±5.3^{a}	74.15 ±5.84^{b}	92.55 ±2.83^{c}	112.05 ±4.6^{d}	117.57 ±4.97^{d}	121.95 ±6.44^{d}
Muscle	2.27 ±0.64^{a}	4.75 ±0.25^{b}	5.55 ±0.55^{bc}	8.41 ±0.65^{d}	7.48 ±0.4^{d}	6.9 ±0.65^{cd}

In our study, two yellow bands from the extraction of fish skin were eluted by the mixture of hexane and acetone (3:1) as a mobile phase in the TLC plate (Figure 2a). Meanwhile, three red bands were separated by the mixture of hexane, petroleum ester and acetone (6:1:2). It is suggested that all three types of astaxanthin (free, mono and diester astaxanthin) were found in the skin of false clownfish when compared with the standard astaxanthin on the TLC plate. The R_f value of diester astaxanthin was higher than that of monoester astaxanthin, while the R_f value of free astaxanthin was the lowest (Figure 1a, b, c). The compositional analysis of fish skin from the TLC plate suggests that *β*-carotene from red bell peppers can both be directly accumulated and/or converted to astaxanthin in fish (Yuangsoi et al 2010; Sathyaruban et al 2021). The strong yellow band (R_f = 0.13) was observed in fish, but was not seen in the *Capsicum* extracts. It could be that zeaxanthin or lutein were synthesized from other carotenoids present in red bell peppers within fish. Lutein is common in freshwater fish and is also widely found in many marine species (Gupta et al 2007). Additionally, zeaxanthin has been observed to be converted from astaxanthin by some fish species (Maoka 2011). Based on the findings, semi-refined carotenoids extracted from red bell peppers using green solvents such as ethanol or vegetable oil have been demonstrated to be safe for animals. Compared to some previous studies, carotenoids isolated from raw materials by organic solvents such as hexane, ether ethyl, and toluene are of great concern to animals due to the bioaccumulation of chemical residues. The results of our study indicated that the diets with carotenoids (in semi-refined form) from red bell peppers significantly improved the skin pigmentation of false clownfish.

There is still some controversy on the role of carotenoids in fish growth. Several studies have shown positive influences, whereas others have found no effects. It is thought that the growth of the false clownfish during culture is influenced by multiple factors such as nutritional ingredients (protein, lipid, carbohydrate, premix vitamins and minerals), feeding regime, and rearing conditions. Our results showed that carotenoids from red bell peppers helped improve the growth parameters of fish.

These results are supported by previous studies highlighting that specific growth rates and weight gain (%) varied significantly ($p<0.05$) among all treatments, and the highest values of SGR and WG were observed in the groups with added paprika oleoresin for *A. ocellaris* (Ebeneezar et al 2020). Paprika powder in a diet (3%) for benni fish (*Mesopotamichthys sharpeyi*) produced significantly higher weight gain and SGR than the control group ($p<0.05$) (Maniat et al 2014). A significant difference in weight, length and condition factor ($p<0.05$) was seen in rainbow trout (*Oncorhynchus mykiss*) fed the diets with and without red peppers (Talebi et al 2013).

Our study found that skin color intensity correlated with the serial levels of dietary carotenoids and the optimal range of carotenoids for enhancing the appearance of false clownfish was between 0.9-1.5 g kg^{-1}. These results are consistent with previous studies demonstrating that natural carotenoids play a vital role in color expression of several fish species. Skin color intensity (the redness) of clownfish *A. ocellaris* was improved with carotenoid-supplemented diets from paprika oleoresin (20 g kg^{-1}) (Ebeneezar et al 2020). It was reported that red peppers significantly enhanced the skin coloration in blue streak hap (*Labidochromis caeruleus*) (50 g kg^{-1} feed) (Yılmaz & Ergün 2011), jewel cichlid (*Hemichromis guttatus*) (3–15% feed) (Yigit et al 2021), pale chub (*Zacco platypus*) (8% paprika) (Lee et al 2010), and juvenile olive flounder (*Paralichthys olivaceus*) (100–200 mg kg^{-1} feed) (Pham et al 2014).

It is found that no significant differences in the lightness (L^*) values were found over all groups supplemented with carotenoids ≤0.9 g kg^{-1}. With higher concentrations of carotenoids, this value tended to decrease. The yellowness (b^*) values slightly increased with the rise in dietary carotenoid levels, except for the dietary carotenoid levels of 1.2–1.5 g kg^{-1}. These results are consistent with previous findings by Pham et al (2014) that lightness (L^*) and yellowness (b^*) were not affected by dietary carotenoid levels for juvenile olive flounder, while Boonyapakdee et al (2015) reported that there was an unpredictable color pattern of L^* and b^* values in carp (*Cyprinus carpio*).

Our results highlight that the accumulation of carotenoids in the skin was much higher than in the whole body and muscles. There was a strong correlation between color expression and total carotenoid content of fish skin with different carotenoid levels. It could be that the range of the dietary carotenoids (0.9–1.5 g kg^{-1} feed) was suitable for improving the skin pigmentation of the false clownfish. Our data was supported by the findings of Ho et al (2013), who discovered that an increase in dietary carotenoid concentration led to a rise in total skin carotenoid concentration. Furthermore, *A. ocellaris* has also been shown to successfully incorporate other carotenoids into its skin under captive conditions, with high values of carotenoids found in paprika and a mixture of paprika (6.78 and 6.97 μg g^{-1} tissue, respectively) (Ebeneezar et al 2020). The total carotenoid content in fish (4.25-7.68 mg kg^{-1} tissue) revealed that rose, hibiscus, marigold and carrot diets can improve the appearance of this species (Ramamoorthy et al 2010).

In other species, high values of carotenoids (0.3 mg g^{-1} tissue) were reported in guppy fish with a mixture of tomato, carrot and red bell pepper at a concentration of 50 mg kg^{-1} feed (Mirzaee et al 2012). Wassef et al (2010) showed that the carotenoids from red pepper in the diets (3 g kg^{-1} feed) helped improve the carotenoid accumulation (1.95 μg g^{-1} dry tissues) in gilthead seabream (*Sparus aurata*). Sources rich in lutein or β-carotene were still suitable for improving fish coloration due to their ability to convert lutein and β-carotene to astaxanthin in fish (Yuangsoi et al 2010). In large yellow croaker (*Larimichthys croceus*), higher carotenoid contents were found in the dorsal and ventral skin of fish fed with xanthophylls compared to those fed with astaxanthin, regardless of dietary supplemental levels (García-Romero et al 2014). With juvenile olive flounder, the supplementation of carotenoids at 100 mg kg^{-1} of feed was suitable for the carotenoid requirement, regardless of sources (paprika or *Haematococcus pluvialis*) (Pham et al 2014).

Conclusions. The results indicate that the suitable dietary carotenoid intake was 0.9 g kg^{-1} over a rearing period of 75 days. The high values of color expression (a^* value) and total carotenoids in tissue were 18.59±0.48 and 112.05±4.6 μg g^{-1}, respectively. The application of natural carotenoids from red bell peppers and other carotenoids from aquatic

and agricultural by-products is much more cost-effective, sustainable, and healthier than the use of non-natural carotenoids in aquaculture. The outcomes of our study have identified red bell peppers as a valuable source of natural carotenoids for improving both red pigmentation and the growth performance in farmed false clownfish.

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Conflict of Interest. The authors declare that there is no conflict of interest.

References

Arimboor R., Natarajan R. B., Menon K. R., Chandrasekhar L. P., Moorkoth V., 2015 Red pepper (*Capsicum annuum*) carotenoids as a source of natural food colors: analysis and stability—a review. Journal of Food Science and Technology 52(3):1258-1271.

Boonyapakdee A., Pootangon Y., Laudadio V., Tufarelli V., 2015 Astaxanthin extraction from golden apple snail (*Pomacea canaliculata*) eggs to enhance colours in fancy carp (*Cyprinus carpio*). Journal of Applied Animal Research 43(3):291-294.

Dang T. T., Bowyer M. C., Van Altena I. A., Scarlett C. J., 2018 Optimum conditions of microwave-assisted extraction for phenolic compounds and antioxidant capacity of the brown alga *Sargassum vestitum*. Separation Science and Technology 53(11):1711-1723.

Das A., Biswas S., 2016 Carotenoids and pigmentation in ornamental fish. Journal of Aquaculture and Marine Biology 4(4):00093.

Ebeneezar S., Prabu D. L., Chandrasekar S., Tejpal C., Madhu K., Sayooj P., Vijayagopal P., 2020 Evaluation of dietary oleoresins on the enhancement of skin coloration and growth in the marine ornamental clown fish, *Amphiprion ocellaris* (Cuvier, 1830). Aquaculture 529:735728.

García-Chavarría M., Lara-Flores M., 2013 The use of carotenoid in aquaculture. Research Journal of Fisheries and Hydrobiology 8(2):38-49.

García-Romero J., Ginés R., Izquierdo M., Haroun R., Badilla R., Robaina L., 2014 Effect of dietary substitution of fish meal for marine crab and echinoderm meals on growth performance, ammonia excretion, skin colour, and flesh quality and oxidation of red porgy (*Pagrus pagrus*). Aquaculture 422-423:239-248.

Gouveia L., Rema P., 2005 Effect of microalgal biomass concentration and temperature on ornamental goldfish (*Carassius auratus*) skin pigmentation. Aquaculture Nutrition 11(1):19-23.

Gupta S., Jha A., Pal A., Venkateshwarlu G., 2007 Use of natural carotenoids for pigmentation in fishes. Natural Product Radiance 6(1):46-49.

Hernández-Ortega M., Ortiz-Moreno A., Hernández-Navarro M. D., Chamorro-Cevallos G., Dorantes-Alvarez L., Necoechea-Mondragón H., 2012 Antioxidant, antinociceptive, and anti-inflammatory effects of carotenoids extracted from dried pepper (*Capsicum annuum* L.). Journal of Biomedicine and Biotechnology 2012:524019.

Ho A. L., O'Shea S. K., Pomeroy H. F., 2013 Dietary esterified astaxanthin effects on color, carotenoid concentrations, and compositions of clown anemonefish, *Amphiprion ocellaris*, skin. Aquaculture International 21(2):361-374.

Kim J. S., An C. G., Park J. S., Lim Y. P., Kim S., 2016 Carotenoid profiling from 27 types of paprika (*Capsicum annuum* L.) with different colors, shapes, and cultivation methods. Food Chemistry 201:64-71.

Lee C. R., Pham M. A., Lee S. M., 2010 Effects of dietary paprika and lipid levels on growth and skin pigmentation of pale chub (*Zacco platypus*). Asian-Australasian Journal of Animal Sciences 23(6):724-732.

Liu X., Mai K., Liufu Z., Ai Q., 2014 Effects of dietary protein and lipid levels on growth, nutrient utilization, and the whole-body composition of turbot, *Scophthalmus maximus*, Linnaeus 1758, at different growth stages. Journal of the World Aquaculture Society 45(4):355-366.
Luo M., Lu G., Yin H., Wang L., Atuganile M., Dong Z., 2021 Fish pigmentation and coloration: Molecular mechanisms and aquaculture perspectives. Reviews in Aquaculture 13(4):2395-2412.
Maiti M. K., Bora D., Nandeesha T. L., Sahoo S., Adarsh B. K., Kumar S., 2017 Effect of dietary natural carotenoid sources on colour enhancement of Koi carp, *Cyprinus carpio* L. International Journal of Fisheries and Aquatic Studies 5(4):340-345.
Maniat M., Ghotbeddin N., Ghatrami E. R., Azaram H. M., 2014 Effect of different levels of paprika on some growth factors, survival and biochemical body composition of benni fish (*Mesopotamichthys sharpeyi*). Academic Journal of Science 3(3):223-229.
Maoka T., 2011 Carotenoids in marine animals. Marine Drugs 9(2):278-293.
McLean E., 2021 Fish tank color: An overview. Aquaculture 530:735750.
Mirzaee S., Shabani A., Rezaee S., Hosseinzadeh M., 2012 The effect of synthetic and natural pigments on the color of the guppy fish (*Poecilia reticulata*). Global Veterinaria 9(2):171-174.
Nankervis L., Cobcroft J. M., Nguyen N. V., Rimmer M. A., 2022 Advances in practical feed formulation and adoption for hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) aquaculture. Reviews in Aquaculture 14(1):288-307.
Pham M. A., Byun H. G., Kim K. D., Lee S. M., 2014 Effects of dietary carotenoid source and level on growth, skin pigmentation, antioxidant activity and chemical composition of juvenile olive flounder *Paralichthys olivaceus*. Aquaculture 431:65-72.
Ramamoorthy K., Bhuvaneswari S., Sankar G., Sakkaravarthi K., 2010 Proximate composition and carotenoid content of natural carotenoid sources and its colour enhancement on marine ornamental fish *Amphiprion ocellaris* (Cuveir, 1880). World Journal of Fish and Marine Sciences 2(6):545-550.
Rhim J. W., Hong S. I., 2011 Effect of water activity and temperature on the color change of red pepper (*Capsicum annuum* L.) powder. Food Science and Biotechnology 20(1):215-222.
Sathyaruban S., Uluwaduge D. I., Yohi S., Kuganathan S., 2021 Potential natural carotenoid sources for the colouration of ornamental fish: a review. Aquaculture International 29(4):1507-1528.
Schweiggert R., Carle R., 2017 Carotenoid deposition in plant and animal foods and its impact on bioavailability. Critical Reviews in Food Science and Nutrition 57(9):1807-1830.
Singh M. K., Borgohain P., Kaur K., Gogoi S., 2021 Emergence of plants in fish pigmentation. Agricultural Research 10(4):535-542.
Sköld H. N., Aspengren S., Cheney K. L., Wallin M., 2016 Fish chromatophores—from molecular motors to animal behavior. International Review of Cell and Molecular biology 321:171-219.
Talebi M., Khara H., Zoriehzahra J., Ghobadi S., Khodabandelo A., Mirrasooli E., 2013 Study on effect of red bell pepper on growth, pigmentation and blood factors of rainbow trout (*Oncorhynchus mykiss*). World Journal of Zoology 8(1):17-23.
Tran D. V., Dang T. T., Cao T. T., Hua N. T., Pham H. Q., 2022 Natural astaxanthin extracted from shrimp waste for pigment improvement in the Orange clownfish, *Amphiprion percula*. Aquaculture Research 53(11):4190-4198.
Wassef E. A., Chatzifotis S., Sakr E. M., Saleh N. E., 2010 Effect of two natural carotenoid sources in diets for gilthead seabream, *Sparus aurata*, on growth and skin coloration. Journal of Applied Aquaculture 22(3):216-229.
Yi X., Shen H., Li J., Wei Z., Shentu J., Zhang W., Mai K., 2018 Effects of dietary vitamin E and astaxanthin on growth, skin colour and antioxidative capacity of large yellow croaker *Larimichthys crocea*. Aquaculture Nutrition 24(1):472-480.
Yigit N. O., Ozdal A. M., Koca S. B., Ozmen O., 2021 Effect on pigmentation, growth and liver histopathology of red pepper (*Capsicum annuum*) added in different ratios to jewel cichlid (*Hemichromis guttatus*) diet. Aquaculture Research 52(11):5579-5584.

Yılmaz S., Ergün S., 2011 Effect of red pepper (*Capsicum annum*) on pigmentation of blue streak hap (*Labidochromis caeruleus*). The Israeli Journal of Aquaculture-Bamidgeh 63:633-638.
Yuangsoi B., Jintasataporn O., Areechon N., Tabthipwon P., 2010 The use of natural carotenoids and growth performance, skin pigmentation, and immune response in fancy carp (*Cyprinus carpio*). Journal of Applied Aquaculture 22(3):267-283.
Zeb A., Murkovic M., 2010 Thin-layer chromatographic analysis of carotenoids in plant and animal samples. JPC–Journal of Planar Chromatography–Modern TLC 23:94-103.
Zhang C., Yao W., Wen D., Li X., Wu S., Leng X., 2020 Dietary *Adonis. aestivalis* extract improved the flesh pigmentation, antioxidative status and shelf-life of rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition 26(6):2032-2042.
*** AOAC, 2005 Official methods of analysis. 18th Edition. Association of Official Analytical Chemists International, USA.
*** Decree 32/2006/ND-CP, 2006 Management of endangered, precious, and rare species of wild plants and animals. Hanoi, Vietnam.
*** The Law of Animal Husbandry of Vietnam, 2018. Available at: https://www.economica.vn/Content/files/LAW%20%26%20REG/Law%20on%20Animal%20Hus bandry%202018.pdf

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