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Dietary supplementation of astaxanthin modulates skin color and liver antioxidant status of giant grouper (*Epinephelus lanceolatus*)

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ABSTRACT

Giant grouper (*Epinephelus lanceolatus*) is an emerging aquaculture species in Southeast Asia and Australia with limited knowledge of its nutrient requirements and effects of supplements on its physiology. The present study investigated the effects of astaxanthin, vitamin E, and combinations on growth performance, body coloration, and the antioxidant status of juvenile giant grouper. Nine isonitrogenous (crude protein = 65 % ± 0.7 %) and isolipidic (crude lipid = 10 % ± 0.3 %) diets were formulated using a 3 × 3 factorial design, including three levels astaxanthin (0, 75, and 150 mg/kg) and vitamin E (0, 250, and 500 mg/kg), respectively. Each of the nine diets was fed to triplicate groups of 15 giant grouper (18.04 ± 0.92 g) for 30 days. Giant grouper fed the different diets exhibited no significant differences ($p > 0.05$) in specific growth rate (4.87 %/day - 5.21 %/day). However, dietary astaxanthin supplementation significantly enhanced the redness (a^*), yellowness (b^*b^*), chroma, and hue values of the fin, regardless of the dose supplemented. Giant grouper fed astaxanthin at 75 and 150 mg/kg diet were more yellow and had three times higher b^* values than fish fed non-supplemented diets. Further, total antioxidant capacity (TAC; mmol Trolox equivalent) in liver tissues was significantly increased in fish fed any of the astaxanthin-supplemented diets ($p \leq 0.05$). In contrast, TAC levels were not affected by vitamin E supplementation. Malondialdehyde (MDA) levels were not significantly ($p > 0.05$) affected by astaxanthin or vitamin E. Findings from this study will contribute toward a better understanding of the dietary effects of antioxidant and pigment in juvenile giant grouper. We present that dietary treatment can modulate giant grouper pigmentation and may be used in the live fish trade. Further, this study contributes to narrowing the knowledge gap in formulating appropriate diets for giant grouper, which to date is fed diets formulated for other species.

1. Introduction

Giant grouper, *Epinephelus lanceolatus* (Bloch, 1790; hereafter referred to as 'GG'), has become a candidate for aquaculture due to its fast growth rates, robustness, and attractive market price (Sung et al., 2019, Dennis et al., 2020). Despite its high market demand and suitability for aquaculture, the specific nutritional requirements of GG remain largely unknown (Nocillado et al., 2021). To date, research on species of the genus *Epinephelus* spp. has focused primarily on the orange-spotted grouper (*Epinephelus coioides*) and the hybrid of giant grouper and tiger grouper (*Epinephelus fuscoguttatus x E. lanceolatus*) (Nankervis et al., 2021; Rimmer and Glamuzina, 2019). GG has a characteristic yellow and black coloration that often fades to shades of gray in farmed fish. Color fading is common in many other pigmented aquaculture species due to low levels of carotenoids in their diet, resulting in reduced market appeal (Wade et al., 2017; Yi et al., 2014; Asche et al., 2001). Aquatic animals are unable to synthesize carotenoids *de novo* (Fang et al., 2019). Some fish species, including salmon, are able to deposit various pigments in their muscle tissue when supplied or acquired through the diet (Nakano et al., 1995; Lorenz and Cysewski, 2000; Viera et al., 2018) and others, such as marine ornamental fish, deposit carotenoids in their skin, modulating the skin surface color and changing the fish appearance (Kalinowski et al., 2007; Yi et al., 2014). In fish, body coloration are used for camouflage, behavioral signaling, and mating (De Carvalho and Caramujo, 2017). Further, an enhanced body coloration correlates with fitness measures, higher social status, and lower parasite load (reviewed in Sefc et al., 2014).

Astaxanthin (3,30′-dihydroxy-β, β′-carotene-4,4′-dione) is a

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carotenoid pigment of red coloration and is soluble in lipid (Ambati et al., 2014). Astaxanthin is widely used as a functional feed ingredient in aquaculture, providing pink-red coloration to the integumentary system (e.g., skin and flesh) of finfish (e.g., in salmonids) and the exoskeleton of crustaceans (Bell et al., 2000). Supplementation of astaxanthin to aquafeed is primarily aimed at modulating color; however, research has shown that astaxanthin can also improve growth, survival, and feed conversion ratio, not only in fish whose coloration is modulated, but also in fish where it has no color-modulating effect (Lim et al., 2019). Further, astaxanthin serves as a metabolic precursor (provitamin) of vitamin A in salmonid fish (Christiansen and Torrissen, 1995), a potent antioxidant (Bell et al., 2000), and a means of improving resistance to stress and disease in fish (Christiansen et al., 1995a, 1995b; Galindo-Villegas et al., 2006).

Vitamin E is a standard antioxidant added to commercial aquafeed as α-tocopherol (α-TOH), whose bioactivity exceeds that of other vitamin E homologs (Hamre, 2011). Similar to astaxanthin, adequate levels of vitamin E in feeds are found to promote growth (Peng et al., 2009; Abdel-Hameid et al., 2012) in fish and increase resistance to stress and diseases (Sahoo and Mukherjee, 2002; Puangkaew et al., 2004; Li et al., 2013). In *Epinephelus malabaricus* vitamin E improved growth (Lin and Shiau, 2005), and in salmonoids, it had synergetic effects with astaxanthin as an antioxidant tested both in vitro and in vivo (Christiansen et al., 1995a, 1995b; Bell et al., 2000).

Understanding the effects of dietary inclusion levels and interactions of these two supplements is critical to further optimize the growth, feed utilization, and welfare of GG. This information may be used for the efficient formulation of antioxidants in GG aquafeed. Although the supplementation of astaxanthin and vitamin E to the diet is reported to exert many benefits, to our best knowledge, neither the individual effects nor their combination has been investigated in GG.

The aim of this study is to measure the individual and synergistic effects of dietary astaxanthin and vitamin E supplementation on the growth, coloration, and antioxidant status on juvenile GG.

2. Materials and methods

2.1. Ethics statements

All handling of fish was conducted following the "Australian Code for the care and use of animals for the scientific purposes". James Cook University Animal Ethics Committee approval number is A2708.

2.2. Experimental design

This study applied a 3 × 3 factorial design to evaluate the effects of different dietary supplementation levels of astaxanthin (AX), vitamin E (VE), and their combination (AX+VE) on the performance of giant grouper. Nine isonitrogenous and isolipidic diets were formulated to be identical except for three levels of AX supplementation (0, 75, and 150 mg/kg) and three levels of VE supplementation (0, 250, and 500 mg/kg) where the supplemented nutrient was added at the expense of wheat flour (Table 1 and Table 2).

2.3. Diet manufacture and nutrient composition

Astaxanthin and vitamin E were individually premixed with wheat flour to ensure consistent distribution (Hobart A200N Planetary Mixer, Hobart, UK). Then, all other dry ingredients were added and mixed again before adding oil and sufficient water for pelleting. The semi-wet dough was pelleted through a 3 mm die (Hobart A120 Planetary Mixer with mincer attachment, Hobart, Australia) and then steamed at 100 °C for 10 min and dried at 50 °C for 12 h. Dried pellets were sieved to remove fines and stored at − 18 °C.

Crude lipid and vitamin E contents (tocopherol) were analyzed by Symbio Laboratories (QLD, Australia) using ether extraction (Soxhlet) and high-performance liquid chromatography (HPLC) methods, respectively. Moisture contents were determined by oven-drying the samples at 105 °C to constant weight. The nitrogen content was determined using a Costech elemental analyzer fitted with a zero-blank autosampler and multiplied by 6.25 to calculate crude protein content.

Table 1
Experimental design and supplementation levels of astaxanthin (AX) and vitamin E (VE) applied in this study.

AX (mg/kg)[1,3]	VE (mg/kg)[2]		
	0	**250**	**500**
0	control	VE250	VE500
75	AX75	AX75 + VE250	AX75 + VE500
150	AX150	AX150 + VE250	AX150 + VE500

1added as CAROPHYLL® Pink 10 %–CWS containing a minimum 11.1 g/kg (DSM Certificate of Analysis) of unesterified chemically synthesized astaxanthin in a corn starch-coated matrix of lignosulfonate and corn oil (DSM Nutritional Products Ltd, France).
2added as ROVIMIX® E-50 Adsorbate, a free-flowing powder of stabilized vitamin E consisting a minimum of 50.5 g/kg (DSM Certificate of Analysis) of DL-α-tocopheryl acetate adsorbed on silicic acid (DSM Nutritional Products Ltd, China).

2.4. Feeding trial

2.4.1. Experimental setup

Each diet was randomly assigned to triplicate rectangular polyethylene tanks (48 × 37 × 26 cm^3; water volume = 45 L). All tanks were supplied with a seawater recirculation system at a flow rate of 7.5 L/min. The temperature was maintained at 28 ± 0.5 °C, salinity 32–35 g/L and dissolved oxygen at 95–110 % saturation with the addition of technical oxygen. Photoperiod was controlled by automatic lights at 12 h L: 12 h D. Total ammonia nitrogen (TAN; NH_4^+-N/NH_3-N), nitrite (NO_2^--N), and nitrate (NO_3^--N) were maintained below 3, 5, and 250 mg/L, respectively.

2.4.2. Fish husbandry and growth measurement

Juvenile GG were sourced from a commercial hatchery (The Company One, Cairns, Australia). The animals were subjected to 250 ppm formalin bath for 45 min upon arrival before being introduced to the recirculating aquaculture system (RAS) facility as a standard disease control procedure. Fish were acclimated in the experimental tanks and were hand-fed to apparent satiation with a commercial diet (Marine Float, Ridley Aquafeed, Australia; CP = 45 %, CL = 20 %) twice daily at 09:00 and 16:30 for four weeks before the feeding experiment.

Before undertaking any measurement procedures, all fish were fasted for approximately 24 h. The animals were gently hand-caught using scoop nets and were anaesthetized using iso-eugenol (AQUI-S, New Zealand Ltd) at a concentration of 25 mg/L. Juveniles GG of uniform initial size were individually weighed to the nearest 0.01 g and total length measured to the nearest mm before being allocated to each of the 27 experimental tanks (fish number = 405; 16.42 ± 2.7 g; 91 ± 8 mm). Fish were hand-fed with corresponding experimental diets to apparent satiation twice daily at 08:30 and 16:00 for 30 days. Uneaten feed after each feeding event was collected, oven-dried, and weighed to allow the correction of feed intake. Fish survival and feeding behavior in each tank were monitored daily. Any dead fish were removed immediately and not replaced. All individuals were again measured for body weight and total length at the end of the feeding trial.

2.5. Image collection

In order to determine the whole body and fin coloration, all 404 survived individuals were photographed under identical lighting condition, angle, and object orientation. The fish were photographed using

Table 2
Formulation and composition of the experimental diets.

Ingredients (g/kg feed)	Experimental diets								
	control (basal diet)	AX75	AX150	VE250	VE500	AX75 VE250	AX75 VE500	AX150 VE250	AX150 VE500
Fish meal[1]	525	525	525	525	525	525	525	525	525
Soy protein isolate[2]	118.8	118.8	118.8	118.8	118.8	118.8	118.8	118.8	118.8
Soybean meal[3]	95	95	95	95	95	95	95	95	95
Lupin seed meal[4]	62	62	62	62	62	62	62	62	62
Fish (anchovy) oil[5]	41	41	41	41	41	41	41	41	41
Wheat flour[6]	100	99.25	98.5	99.5	99	98.75	98.25	98	97.5
Gelatine	30	30	30	30	30	30	30	30	30
Soy lecithin[7]	10	10	10	10	10	10	10	10	10
L-lysine HCl[8]	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
DL-methionine[9]	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Vitamin mix[10]	1	1	1	1	1	1	1	1	1
Mineral mix[11]	5	5	5	5	5	5	5	5	5
Choline chloride 70 %	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CAROPHYLL® Pink 10 %–CWS[12]	0	0.75	1.5	0	0	0.75	0.75	1.5	1.5
ROVIMIX® E-50 Adsorbate[13]	0	0	0	0.5	1	0.5	1	0.5	1
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Proximate composition (g/kg DM)									
Crude protein	65.06	65.56	64.19	64.38	66.06	65.19	64.38	64.56	65.44
Crude lipid	10.2	9.7	10.0	10.2	10.1	10.3	10.3	9.7	9.7
Astaxanthin (mg/kg)[14]	0	75	150	0	0	0	75	150	150
Vitamin E (mg/kg)[15]	3.4	7.0	9.0	208.7	443.6	208.6	463.3	227.5	457.5

[1,5]Ridley Aquafeeds, Australia. [2]Riverina, Australia. [3,8]Bulk Nutrients, Australia. [4]The Source Bulk Foods, Australia. [6]Coles, Australia. [7]Dancourt Trading, Australia. [9]Create Your Own Supplements (CYOS), Australia.
[10]RABAR Animal Nutrition, Australia. Vitamin profile (per kg diet): A 3000 IU; D 24 IU; K 10 mg; B1 (Thiamine) 10 mg; B2 (Riboflavin) 20 mg; B3 (Nicotinic acid) 45 mg; B5 (Pantothenic acid) 10 mg; B6 (Pyridoxine) 10 mg; B12 (Cyanocobalamin) 0.05 mg; C 150 mg; Biotin 1 mg; Inositol 250 mg; Folic acid 5 mg; Antioxidant 15 mg; and dextrose was used as a carrier.
[11]Mineral profile (per kg diet): Mg 297 mg; Zn 100 mg; Fe 40 mg; Mg 25 mg; Cu 5 mg; I 4 mg; Co 0.5 mg; Se 0.1 mg.
[12]contain a minimum of 10 % of unesterified synthetic astaxanthin in a corn starch-coated matrix of lignosulfonate and corn oil (DSM Nutritional Products Ltd, France).
[13]sprayed-dried powder consisting of minimal 50 % of DL-α-tocopheryl acetate finely dispersed in a matrix of lignosulfonate and coated with small amounts of silicon dioxide (DSM Nutritional Products Ltd, China).
[14]as added astaxanthin.
[15]as analyzed tocopherol.

a digital camera (Sony A5000 ILCE-5000, Japan) inside a light-proof aluminum box (670 mm length × 600 mm width × 800 mm height) with standardized illumination provided by LED strip lights placed along the upper wall of the box. The light traveled through a diffusion plate before illuminating the bottom plate where the fish were placed. This design prevents the specular light reflection from the base plate and the fish objects. This diffusion plate also shielded the camera's field of view, preventing stray light from the light source from entering the camera lens directly. Light entering the camera lens was entirely reflected from the bottom plate, color chart and fish. All openings were sealable to prevent external light contamination. An opening at the top of the box was filled with the camera lens (focal length = 16 mm) facing perpendicularly downwards, focusing on the objects. The camera exposure setting was set as follows: ISO = 100, aperture = F/10, exposure time = 1/60 s, and a camera max resolution of 19.8 megapixels. The fish were photographed in groups of five. A patch containing standardized colors (X-Rite Pantone, USA) was included in each photograph for calibration in downstream analyses.

2.6. Antioxidant status measurements

The liver tissues samples were collected from three randomly selected fish per tank (81 individuals in total) at the end of the feeding trial. The fish were ethically euthanized using iso-eugenol at 175 mg/L for 20 min. Afterwards, the whole liver was excised, weighed, homogenized on ice, and immediately snap-frozen in liquid nitrogen. The samples were stored at − 80 until analyzed.

The total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in the liver tissue were quantified using commercial kits (CS0790, Sigma-Aldrich, USA) and (MAK085, Sigma-Aldrich, USA), respectively, according to the manufacturer's instructions. Tissue homogenization using silica beads was performed within a pre-chilled stainless-steel block at 20 °C and shaken in a tissue disruptor/bead beater (BioSpec Products Inc., USA). Each liver sample (81 livers) for each parameter of antioxidant status was assayed in duplicate.

2.6.1. Total antioxidant capacity (TAC) assay

Tissue samples (~100 mg) were prepared following the manufacturer's instructions. Tissue were homogenized in the assay buffer and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant (10 μL) was collected and kept on ice until assayed in a 96-well microplate according to the kit instructions. In brief, the assay is based on the formation of ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which then oxidizes ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) to produce a radical cation ($ABTS^{\bullet+}$). This radical cation is a green soluble chromogen causing green coloration in the test sample, inversely proportional to antioxidant level, and can be read in a microplate spectrophotometer at 405 nm (EnSpire® model 2300, PerkinElmer, USA). Trolox™, a water-soluble vitamin E analog, was used as a standard or control antioxidant. The values of total antioxidant capacity were calculated from the standard curve of known concentrations and expressed as mM relative to the concentration of the Trolox standard.

2.6.2. Malondialdehyde (MDA) assay

Tissue samples (~10 mg) were prepared following the manufacturer's instructions. Samples were homogenized in the lysis buffer and centrifuged at 13,000 × *g* for 10 min to remove insoluble material. The supernatant (200 μL) was transferred into a microcentrifuge tube and assayed according to the kit instruction. In brief, the MDA in the sample is quantified through their reaction with thiobarbituric acid (TBA) at 95 °C for 1 h to form a colorimetric product proportional to the MDA

present. This colorimetric product was detected using a microplate spectrophotometer at 532 nm (EnSpire® model 2300, PerkinElmer, USA). The MDA concentration in the samples was calculated from the standard curve of known concentrations and expressed as nmole/mg tissue. The MDA level indicates the lipid peroxidation activity in the sample.

2.7. Image analysis

2.7.1. Whole-body coloration

The method to analyze body coloration was adapted from Weller and Westneat (2019) and Van Belleghem et al. (2018). The raw images (5456 × 3632 pixels; ~20 megabytes each) were calibrated for any variation in illuminance using Adobe Lightroom CC (Adobe Inc., USA). Images of individual fish were obtained by cropping out the fish from the group using CorelDRAW X-7 (Corel, Canada) before the background was removed using Remove.bg (Kaleido, Germany). All pixels of each image were binned into 27 regions in the red, blue, green (RGB) color space using the histogram method as described in Weller and Westneat (2019). The color intensity and relative proportion of these color bins were statistically compared using the color distance metric in a pairwise manner among all individuals. The color distance between two individuals is defined as the formula below calculated using the R package *colordistance* (Weller and Westneat, 2019).

$$\text{color distance} = \sum_{i=1}^{y} \sqrt{(R_i^a - R_i^b)^2 + (G_i^a - G_i^b)^2 + (B_i^a - B_i^b)^2}$$

Where, y = number of bins, R = red value, G = green value, B = blue value.

The color distance value indicates the color similarity or dissimilarity between two individuals, where a high value indicates a high color dissimilarity. Color distance values were further analyzed using principal coordinate analysis (PCoA) to determine whether body coloration was affected by experimental treatments. All quantitative color profiling of each individual fish, and the comparison between them, were performed in RStudio v.4.0.

2.7.2. Caudal fin coloration

Following CIE Lab color space (CIE, 1977), colouration in the caudal fin is described using five parameters: L^*(lightness), a^*(redness/greenness), b^*(yellowness/blueness), chroma ($C_a{}^*{}_b$; intensity and clarity of color), and hue ($H_a{}^*{}_b$; the relationship between redness and yellowness). Considering color may vary across caudal fin, all parameters for each individual (404 fish) were quantified in five sampling areas with the obvious dark spots avoided (Fig. 1). The L^*, a^*, and b^* were quantified using an image analysis software, Image Pro Premier 9 (Media Cybernetics Inc., USA); while the chroma (C_{a^*b}) and hue (H_{a^*b}) were calculated using the obtained a^* and b^* value according to the following formulas (Hunt, 1977).

$$C_{a*b} = \sqrt{(a^*)^2 + (b^*)^2}$$

$$H_{a*b} = tan^{-1}\left(\frac{b^*}{a^*}\right)$$

The average value for each parameter across five areas was calculated to represent the individual fish value; subsequently, the mean value of all individual fish within the same tank was calculated to represent the tank value.

2.8. Data analyses

2.8.1. Growth performance calculations

The raw data of growth performance were recorded in Microsoft Excel, and the below formulas were used to calculate each growth parameter:

$$\text{WG (weight gain, \%)} = \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight (g)}} \times 100\%$$

$$\text{SGR (specific growth rate; \%/day)} = \frac{ln\ \text{final weight (g)} - ln\ \text{initial weight (g)}}{\text{number of feeding days}} \times 100$$

$$\text{CF (condition factor; g/cm}^3\text{)} = \frac{\text{body weight (g)}}{\text{total length (cm)}^3} \times 100$$

$$\text{FI (daily feed intake; \%BW/day)} = \frac{\text{dry feed intake (g)} \times \text{number of feeding days (days)}}{[\text{initial biomass (g)} + \text{final biomass (g)} + \text{dead biomass (g)}] \div 2} \times 100$$

$$\text{FCR (feed conversion ratio)} = \frac{\text{dry feed intake (g)}}{\text{final biomass (g)} + \text{dead biomass (g)} - \text{initial biomass (g)}}$$

$$\text{HSI (hepatosomatic index; \%)} = \frac{\text{liver weight (g)}}{\text{body weight (g)}} \times 100$$

$$\text{Survival (\%)} = \frac{\text{final fish number}}{\text{initial fish number}} \times 100$$

2.8.2. Statistical analyses

Tanks are the experimental unit (replicate) and also the unit of statistical assessment. The results are reported as a treatment mean of the triplicate tanks ± standard deviation (SD). The normality and homogeneity of variance of the response variable (the growth performance, coloration, and antioxidant status) for each combination of independent variables (the supplementation level of astaxanthin, vitamin E, and their combination) were assessed using Shapiro Wilk and Levene's test,

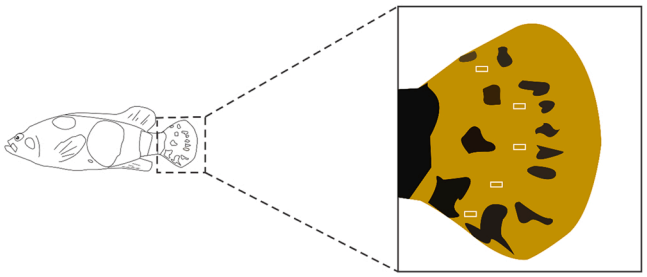

Fig. 1. Five areas of the caudal fin (as indicated by the white rectangles) sampled for the coloration analysis.

respectively. Outliers were identified using boxplot method, the boxplot (x) function, in R and appropriately removed.

A two-way analysis of variance (ANOVA) was used to investigate whether mean differences in response variables existed between treatments. Where two-way ANOVA revealed a single factor was responsible for differences between treatments, with no interaction terms, values were pooled accordingly to allow detection of differences by one-way ANOVA followed with pairwise comparison using Tukey post-hoc test or pairwise t-test. Differences between treatment means were considered significant at $p \leq 0.05$. All statistical analyses were performed in RStudio v.4.0 (RStudio Team, 2019).

3. Results

3.1. Effects of experimental diets on the body and caudal fin coloration

The color distance between each individual in a pairwise manner (404 × 404) across the treatment group is presented by the heatmap plot (Fig. 2A). These color distance scores ranged between 0 and 2.56 (Fig. 2A). The clustering shown in the heatmap is elucidated in the principal coordinate analysis (PCoA) plot (Fig. 2B). The PCoA plot revealed that the two clusters are formed, associated to the presence or absence of astaxanthin supplementation in the diet (Fig. 2B). Furthermore, not a single member from each of these two groups overlapped the region of the other group at a 95 % confidence level, and no other diet-related differences were detected by this color analysis (Fig. 2B). The visual inspection of juvenile giant grouper at the end of the feeding trial clearly indicates that fish fed with the AX supplemented diet had a more pronounced yellow coloration in the fin than the control group (Fig. 3).

The coloration of the caudal fin of juvenile GG fed with different experimental diets is presented in Table 3. No interaction effects of AX with VE were detected ($p > 0.05$; Table 3). The a^* (redness/greenness), b^* (yellowness/blueness), chroma (intensity and clarity) and hue (the

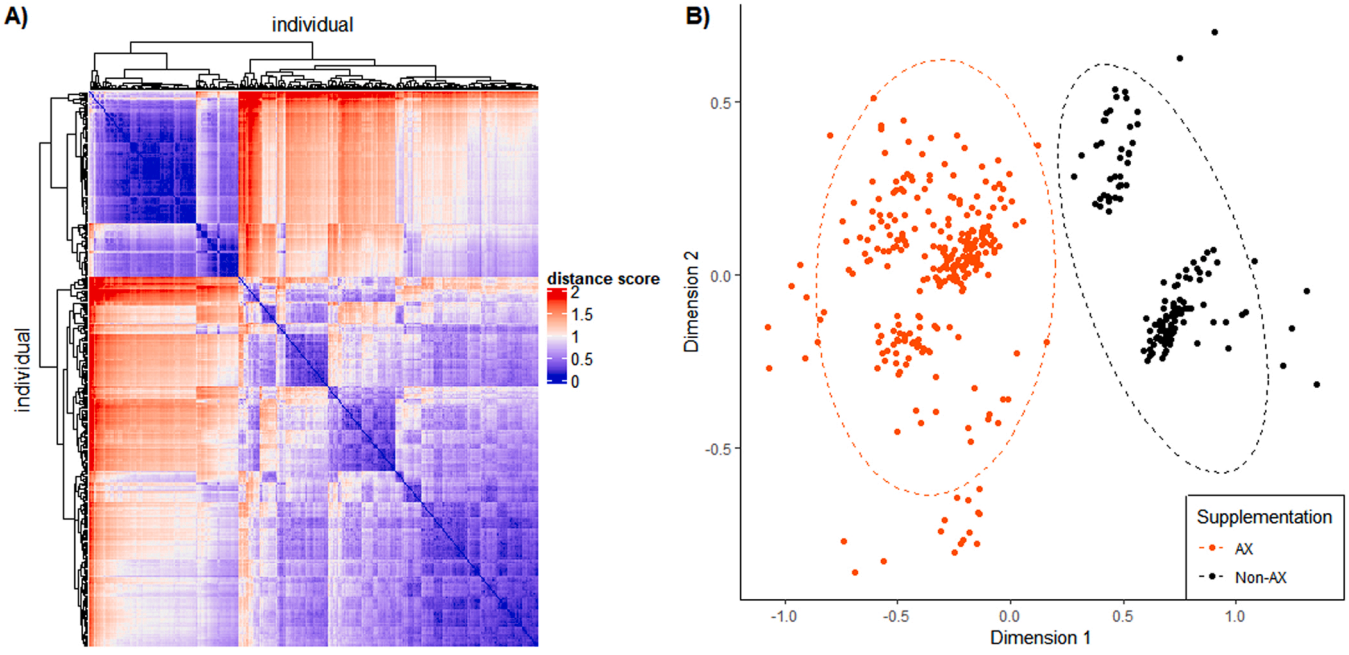

Fig. 2. (A) Heatmap of color distance score resulted from comparing whole-body coloration among 404 individual fish in a pairwise manner (404 × 404). The color of each cell indicates the color distance score between two corresponding individuals; the name of individual fish is not displayed for the purpose of plot visibility. (B) Principal coordinate analysis of the heatmap of color distance score. Each dot represents individual fish, and the distances between dots indicate their color similarity calculated according to the color distance metric. Representative fish from each group is displayed. The confidence ellipses (dash circles) indicate the true population distribution in the bivariate distribution calculated at 95 % confidence level.

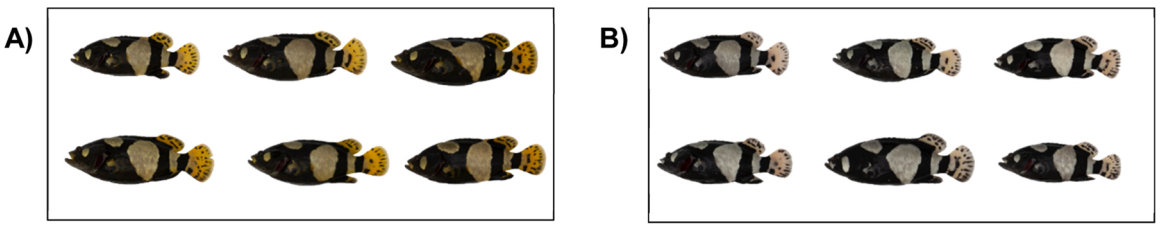

Fig. 3. Photographs showing representative fin coloration of juvenile giant grouper fed with a diet supplemented with (A) 150 mg/kg of AX (AX150), and (B) control (without astaxanthin).

Table 3
Caudal fin coloration of juvenile giant grouper fed with diets supplemented with different levels of astaxanthin, vitamin E, and their combination after a 30-day feeding trial.

Parameters[a,b]	Experimental diets									Two-way ANOVA		
	control	AX75	AX150	VE250	VE500	AX75 + VE250	AX75 + VE500	AX150 + VE250	AX150 + VE500	AX	VE	AX* VE
L	60.12 ± 2.81	58.04 ± 2.26	58.04 ± 1.94	60.32 ± 0.79	59.99 ± 2.66	58.36 ± 1	58.6 ± 0.35	58.78 ± 2.97	59.11 ± 1.33	ns	ns	ns
*a**	4.19 ± 1.36	6.76 ± 0.18	8.12 ± 1.33	4.66 ± 0.19	4.05 ± 0.18	6.38 ± 0.8	6.26 ± 0.58	7.04 ± 0.56	7 ± 0.4	***	ns	ns
*b**	16.88 ± 2.23	51.98 ± 3.87	56.77 ± 3.48	18.4 ± 0.53	16.26 ± 0.21	51.44 ± 2.03	48.6 ± 1.82	52.03 ± 2.14	52.38 ± 3.13	***	ns	ns
Chroma	17.5 ± 2.36	52.49 ± 3.81	57.43 ± 3.61	19.05 ± 0.45	16.82 ± 0.2	51.91 ± 2.08	49.07 ± 1.78	52.58 ± 2.2	52.91 ± 3.05	***	ns	ns
Hue	1.32 ± 0.06	1.43 ± 0.03	1.4 ± 0.03	1.32 ± 0.02	1.33 ± 0.01	1.42 ± 0.04	1.43 ± 0.02	1.44 ± 0.01	1.41 ± 0.06	***	ns	ns

[a] Abbreviations are as follows: *L* = lightness, *a** = redness/greenness, *b** = yellowness/blueness.
[b] Value are presented as mean ± SD from three replicate groups. Different asterisk indicates level of significance of ANOVA test; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = no significant difference ($p > 0.05$).

Table 4
Color parameters in the caudal fin of juvenile giant grouper grouped by different levels of astaxanthin supplementation (0, 75, and 150 mg/kg). Different superscripts ($^{a-c}$) denote statistically significant differences within each row (one-way ANOVA $p \leq 0.05$).

Parameters[1,2]	Pooled group		
	AX0	AX75	AX150
*a**	4.3 ± 0.75[a]	6.46 ± 0.55[b]	7.39 ± 0.93[c]
*b**	17.18 ± 1.49[a]	50.67 ± 2.84[b]	53.73 ± 3.44[b]
Chroma	17.79 ± 1.56[a]	51.16 ± 2.83[b]	54.31 ± 3.51[b]
Hue	1.32 ± 0.03[a]	1.43 ± 0.03[b]	1.42 ± 0.04[b]

[1]Value are presented as mean ± SD from three replicate groups.

relationship between redness and yellowness) values were significantly affected by the dietary supplementation of astaxanthin (Table 3), all values progressively increasing with AX supplementation in the diet (Table 4). The increment was most pronounced in the *b** and chroma value, almost three times higher compared to the non-astaxanthin group (AX0) (Table 4). The increment of *b** value from the AX75 group to the AX150 group was not statistically significant, in term of absolute value, it differed only by + 3.06 points (Table 4).

3.2. Effects of experimental diets on antioxidant status

The two-way ANOVA results indicated that the total antioxidant capacity (TAC) level in the liver tissue was significantly affected only by the dietary supplementation of astaxanthin (Table 5). In particular, the liver samples of the fish group fed with astaxanthin supplemented at 150 mg/kg diet has a slightly higher TAC level (1.61 mM) compared to the control group (1.57 mM) (Table 6). Meanwhile, the malondialdehyde (MDA) levels were not significantly affected by any dietary treatments (Table 5).

3.3. Effects of experimental diet on growth performance, survival, and feed utilization

The proportional weight gain (WG %) of juvenile GG after 30 day-feeding trial ranged between 332 % and 378 % or equal to 3.32 and 3.78 times of initial fish weight, whereas the specific growth rate (SGR) ranged between 4.87 %/day and 5.21 %/day with no significant differences between treatments (Table 7). The survival rates were 100 % in all tanks, except one fish died in the tank within the VE500 group. Similarly, both supplements and their combination did not affect the hepatosomatic index or feed intake of fish ($p > 0.05$; Table 7).

The feed conversion ratio (FCR) ranged from 0.69 to 0.71 and was significantly affected by astaxanthin supplementation ($p \leq 0.05$; Table 8). Compared to the mean of FCR in the group without astaxanthin supplementation (0.69), a slight increase in FCR was recorded in the group fed with astaxanthin supplemented at 75 mg/kg (0.71) and 150 mg/kg (0.71) ($p \leq 0.05$; Table 8). The final condition factor (CF) ranged from 2.10 to 2.23 and was significantly affected by astaxanthin supplementation ($p \leq 0.05$; Table 8). However, no particular trend in

Table 6
The significantly affected means of the antioxidant status parameter in the liver tissue samples of juvenile giant grouper grouped by different levels of astaxanthin supplementation. Different superscripts ($^{a-b}$) denote statistically significant differences within each row (one-way ANOVA $p \leq 0.05$).

Parameters[1,2]	Pooled group		
	AX0	AX75	AX150
TAC (mM)	1.57 ± 0.05[a]	1.60 ± 0.02[a]	1.61 ± 0.02[b]

[1]Value are presented as mean ± SD from replicates ($n = 9$) pooled within different astaxanthin supplementation levels. AX0 consists of control, VE250, and VE500 group; AX75 consists of AX75, AX75 +VE0, and AX75 +VE500 group; and AX150 consists of AX150, AX150 +VE0, and AX150 +VE500 groups.

Table 5
Total antioxidant capacity (TAC) (A) and malondialdehyde (MDA) levels (B) in the liver tissue samples of juvenile giant grouper fed with different dietary supplementation level of astaxanthin, vitamin E, and their combination.

Parameters[1]	Experimental diets									Two-way ANOVA		
	control	AX75	AX150	VE250	VE500	AX75 + VE250	AX75 + VE500	AX150 + VE250	AX150 + VE500	AX	VE	AX* VE
TAC (mM)	1.52 ± 0.04	1.60 ± 0.01	1.61 ± 0.01	1.61 ± 0.02	1.58 ± 0.06	1.59 ± 0.03	1.62 ± 0.02	1.60 ± 0.04	1.61 ± 0.02	*	ns	ns
MDA (nmole/g)	1.82 ± 0.06	1.80 ± 0.07	1.98 ± 0.34	1.87 ± 0.08	1.67 ± 0.17	1.83 ± 0.26	1.88 ± 0.13	1.87 ± 0.11	1.70 ± 0.08	ns	ns	ns

[1]Value are presented as mean ± SD from three replicate groups. Different asterisk indicates level of significancy of ANOVA test; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **ns** = no significant difference ($p > 0.05$).

Table 7
Growth performance, survival, feed utilization, and hepatosomatic indices of juvenile giant grouper fed with a diet supplemented with different levels of astaxanthin, vitamin E, and their combination.

Parameters[1]	Experimental diets									Two-way ANOVA[2]		
	control	AX75	AX150	VE250	VE500	AX75 + VE250	AX75 + VE500	AX150 + VE250	AX150 + VE500	AX	VE	AX*VE
Initial BW (g)	16.5 ± 0.2	16.4 ± 0.3	16.1 ± 0.3	16.3 ± 0.3	16.3 ± 0.1	16.7 ± 0.9	16.4 ± 0.2	16.7 ± 0.3	16.3 ± 0.3	ns	ns	ns
Final BW (g)	72.7 ± 0.2	74.2 ± 1.1	73.4 ± 3.0	77.8 ± 3.0	76.5 ± 2.9	72.9 ± 2.3	73.0 ± 2.5	72.2 ± 4.3	74.3 ± 1.4	ns	ns	ns
WG (%)	342 ± 4	351 ± 20	356 ± 11	378 ± 23	370 ± 18	337 ± 22	344 ± 21	332 ± 30	356 ± 14	ns	ns	ns
SGR (%)	4.95 ± 0.03	5.02 ± 0.02	5.06 ± 0.08	5.21 ± 0.16	5.16 ± 0.13	4.92 ± 0.17	4.97 ± 0.16	4.87 ± 0.23	5.06 ± 0.1	ns	ns	ns
Initial BL (mm)	91 ± 0.6	91 ± 1.6	91 ± 2.3	92 ± 3.2	93 ± 2.2	92 ± 1.4	92 ± 1.0	93 ± 3.0	93 ± 1.9	ns	ns	ns
Final BL (mm)	150 ± 0.4	152 ± 1.7	150 ± 1.1	152 ± 3.3	151 ± 1.5	151 ± 2.1	150 ± 0.7	149 ± 1.6	149 ± 1.0	ns	ns	ns
Final CF (g/cm^3)	2.14 ± 0.03	2.10 ± 0.06	2.19 ± 0.03	2.20 ± 0.03	2.19 ± 0.06	2.12 ± 0.04	2.13 ± 0.07	2.14 ± 0.08	2.23 ± 0.05	*	ns	ns
HSI (%)	2.76 ± 0.15	2.56 ± 0.32	2.6 ± 0.36	2.49 ± 0.2	2.79 ± 0.16	2.33 ± 0.3	2.23 ± 0.19	2.46 ± 0.34	2.70 ± 0.19	ns	ns	ns
FI (%BW/day)	2.9 ± 0.04	3.02 ± 0.01	2.98 ± 0.06	3.01 ± 0.04	2.95 ± 0.05	2.99 ± 0.08	3.02 ± 0.05	3.01 ± 0.05	3.04 ± 0.07	ns	ns	ns
FCR	0.69 ± 0.01	0.71 ± 0	0.7 ± 0.01	0.7 ± 0	0.69 ± 0.01	0.71 ± 0.01	0.72 ± 0.02	0.72 ± 0.01	0.71 ± 0.02	*	ns	ns
Survival (%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	98 ± 3.9	100 ± 0	100 ± 0	100 ± 0	100 ± 0	ns	ns	ns

[1]Abbreviations are as follows: **BW** = body weight; WG = weight gain; **SGR** = specific growth rate; **IBL** = initial body total length; **FBL** = final total body length; **ICF** = initial condition factor; **FCF** = final condition factor; **HSI** = hepatosomatic index; **FI** = feed intake; **FCR** = feed conversion ratio.
[2]Value are presented as mean ± SD from three replicate groups. Different asterisk indicates level of significancy of ANOVA test; * = $p \leq 0.05$; ** = $p \leq 0.01$; ***= $p \leq 0.001$; **ns** = no significant difference ($p > 0.05$).

Table 8
The significantly affected means of juvenile giant grouper's growth performance grouped by different levels of astaxanthin supplementation. Different superscripts ($^{a\text{-}b}$) denote statistically significant differences within each row (1-way ANOVA $p \leq 0.05$).

Parameters[1, 2]	Pooled group		
	AX0	AX75	AX150
Final CF (g/cm^3)	2.18 ± 0.04^{ab}	2.12 ± 0.05^{a}	2.19 ± 0.06^{b}
FCR	0.69 ± 0.01^{a}	0.71 ± 0.01^{b}	0.71 ± 0.02^{b}

[1]Value are presented as mean ± SD from replicates pooled within different astaxanthin supplementation levels. AX0 consists of control, VE250, and VE500 group; AX75 consists of AX75, AX75 +VE0, and AX75 +VE500 group; and AX150 consists of AX150, AX150 +VE0, and AX150 +VE500 groups.
[2]Different superscripts indicate significantly different mean between dietary astaxanthin group ($p \leq 0.05$) tested using Tukey's multiple comparison test.

relationship with the astaxanthin groups was obvious (Table 8).

4. Discussion

4.1. Whole-body and caudal fin coloration

In fish, the deposition of yellow pigments such as tunaxanthin (Miki et al., 1985), lutein, and zeaxanthin (Bjerkeng et al., 2000) are responsible for the yellowness in the integumentary system. In this study, yellowing (b*) of GG fed one of the astaxanthin-enriched diets was up to three times higher than of GG fed the control diet. These results suggest that astaxanthin is metabolized to yellow pigments, as previously demonstrated for rainbow trout (*Oncorhynchus mykiss*) (Schiedt et al., 1985) and Japanese yellowtail (*Seriola quinqueradiata*) (Miki et al., 1985).

In fish and crustaceans, color parameters, e.g., hue, *a**, and *b** correlate strongly positive with carotenoid concentrations deposited in their tissues and are therefore good indicators of deposited carotenoids (Kalinowski et al., 2011; Sun et al., 2012; Fanning et al., 2014). Our results show that both redness and yellowness increased with increasing astaxanthin concentration; however, differences between GG fed the astaxanthin supplemented and non-supplemented diets were more pronounced than between supplementation levels. Moreover, there was no statistical difference in hue value between these two groups. The CIE Lab color space defines hue as the degree to which a color can be distinguished from other colors (Sun et al., 2012). Thus, the insignificant increase (+3.06 points) of yellowness (*b**), no difference in hue, and no cluster differentiation between AX75 and AX150 groups in the PCOA diagram indicate that xanthophores in the skin of juvenile GG were nearly saturated at the lowest supplementation level of 75 mg/kg diet (Kimler and Taylor, 2002).

Consistent with the results of this study, Booth et al. (2004) reported a similar trend of color saturation in red snapper (*Pagrus auratus*) fed diets containing astaxanthin at 36 mg/kg and 72 mg/kg. Dietary carotenoids gradually accumulate in animal tissues over the intake period, and accumulation rates will decline as the concentration in the storage tissue increases towards the saturation point (Choubert, 2010; Safari and Atashi, 2015). The present finding suggests that the supplementation of 75 mg/kg astaxanthin in the diet is sufficient to intensify the yellow color in the caudal fin of juvenile GG. Translating these results into feed formulations may require further investigation into lower levels of astaxanthin or alternative yellow pigments such as leutin, zeaxathin, or raw material rich in these compounds for more cost-effective color manipulations.

There was no interaction between astaxanthin and vitamin E in promoting GG pigmentation, which contrasts with previous studies on rainbow trout, Arctic char, and yellow croaker (Pozo et al., 1988; Bjerkeng et al., 1999; Yi et al., 2018). This synergistic effect may be attributed to the similarity of astaxanthin and vitamin E as a lipid-soluble antioxidant (Machlin and Bendich, 1987), where sufficient vitamin E intake may spare astaxanthin from oxidation and therefore can be readily deposited in the tissue to induce coloration (Yi et al., 2018). The absence of the sparring effect may indicate a limitation in the antioxidant function of vitamin E in GG, consistent with the results from the liver antioxidant capacity analysis.

4.2. Antioxidant status

Astaxanthin is reported to possess 100- to 500-fold higher antioxidant activity than other antioxidants, e.g., α-tocopherol (vitamin E) and β-carotene (Naguib, 2000), which is consistent with the current results showing that GG fed astaxanthin at 150 mg/kg diet had higher total hepatic antioxidant capacity (TAC) than the control and vitamin E (VE) group. Astaxanthin's ability to serve as a powerful antioxidant is attributed to its molecular structure, which contains hydroxyl (OH) and

keto-moieties (C═O) on each ionone ring (Higuere-Ciapara et al., 2006; Hussein et al., 2006). This structure allows astaxanthin to donate electrons and effectively quench reactive oxygen species (ROS), e.g., $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}HO$ (Higuere-Ciapara et al., 2006; Hussein et al., 2006). Vitamin E supplementation did not increase TAC levels, despite the analysis being based on Trolox equivalent, which is a vitamin E analog. Since approximately three-quarters of final fish weight was due to weight gain during the feeding experiment, it is unlikely that the initial body stock of antioxidants affected the measured TAC in this study.

Malondialdehyde (MDA) is a by-product of lipid peroxidation commonly used as an indicator of oxidative processes in biological systems (Del Rio et al., 2005). Evoked oxidative stress has been shown to correlate with growth and immune response suppression (Long et al., 2019). Therefore, the balance of ubiquitous ROS is critical to ensure optimal animal growth and health. The statistically similar MDA levels across treatments, irrespective of the dietary antioxidant level, indicate that juveniles GG were not under oxidative stress. Under adverse environmental conditions such as temperature stress (Cheng et al., 2018), the need for antioxidant supplementation may increase, which is an avenue for further research in this area.

4.3. Growth performance, survival, and feed utilization

The present study did not find any effect of astaxanthin, vitamin E, or their combination on growth, survival or feed utilization of juvenile GG. Our finding contrasts with previous studies that have shown positive effects of astaxanthin supplementation on growth performance and feed utilization in Atlantic salmon (*Salmo salar*) (Christiansen et al., 1995a, 1995b, 1994), Atlantic cod (*Gadus morhua*) (Hansen et al., 2016), pufferfish (*Takifugu obscurus*) (Cheng et al., 2018), yellow catfish (*Pelteobagrus fulvidraco*) (Liu et al., 2019), large yellow croaker (*Larimichthys croceus*) (Liu et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Bazyar Lakeh et al., 2010), and red porgy (*Pagrus pagrus*) (Kalinowski et al., 2011). The growth-promoting effect of astaxanthin is due to its speculative positive roles on the intermediary metabolism, e.g., ATP generation (Segner et al., 1989; Tacon, 1981), ability to enhance growth hormone (Lim et al., 2019), and potent antioxidant properties which inactivate harmful ROS and optimizes physiological functions, particularly under adverse conditions (Liu et al., 2019).

Astaxanthin also could enhance the activity of digestive enzymes in prawns, thereby increasing feed digestibility and utilization (Niu et al., 2014). In contrast, this study found that astaxanthin supplementation slightly reduced feed utilization, i.e., increased FCR. While the reduction of feed utilization was statistically significant, the degree of change between treatments (−0.02 point) was so small as to be irrelevant in practice. This reduced feed utilization may not reflect a low metabolic cost associated with the conversion of astaxanthin to yellow carotenoids but is more likely an artifact of analytical precision, with nearly identical feed conversion values in many of the experimental tanks, provoking a Type I error.

To the best of our knowledge, the exact mechanism of astaxanthin to improve somatic growth has not been well elaborated. Moreover, the positive effects of astaxanthin on growth performance, feed utilization, and survival were not consistently reported across studies. In agreement with our finding, many other studies found no significant effects of astaxanthin supplementation, e.g., in coral trout (Zhu et al., 2022 [0–173 mg/kg diet]), blood parrotfish (Micah et al., 2022 [450 mg/kg diet]), rainbow trout (Hart and Colombo, 2022 [17–60.6 mg/kg diet]; Yadollahi et al., 2021 [50 mg/kg diet]), Atlantic salmon (Olsen and Baker, 2006 [55 mg/kg diet]), yellow croaker (Luo et al., 2020 [80 mg/kg diet]; Yi et al., 2014 [75 mg/kg diet]), koi (Sun et al., 2012 [150 mg/kg diet]), olive flounder (Pham et al., 2014 [0–200 mg/kg diet]), Australian snapper (Doolan et al., 2008 [60 mg/kg diet]), gilthead seabream (Gomes et al., 2002 [40 mg/kg diet]), characins (Wang et al., 2006 [40 mg/kg diet]), and red porgy (Kalinowski et al., 2005 [38 mg/kg diet]; Tejera et al., 2007 [27–68 mg/kg diet]).

Furthermore, this study did not observe signs of vitamin E deficiency in fish fed with the control diet (analyzed vitamin E content: 3.4 mg/kg diet in dry matter basis), such as reduced growth (Yi et al., 2018; Niu et al., 2014; Zhou et al., 2013; Sau et al., 2004), muscular atrophy, and dyspigmentation (Chen et al., 2004; Kocabas and Gatlin III, 1999). This finding could indicate that the raw materials used in this study may have provided sufficient vitamin E to prevent these symptoms. Similarly, the absence of growth reduction even in fish fed with vitamin E-poor diet has previously been reported in several studies, e.g., in gilthead sea bream (Montero et al., 2001), golden shiner (*Notemigonus crysoleucas*) (Chen et al., 2004), turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) (Tocher et al., 2002) and meager (*Argyrosomus regius*) (Lozano et al., 2017). Dietary antioxidants, such as vitamin E, are reported to improve growth only in fish exposed to high oxidative risk, such as in stressful environments (Montero, 2001) and fed a highly oxidized diet (Gao et al., 2012; Tocher et al., 2003).

The effects of astaxanthin and vitamin E on fish growth, feed utilization, and survival are inconsistent across previous studies, even when the same species were studied. This suggests that the effects of dietary antioxidants likely depend on other factors. It has been reported that inclusion level and stress status (Liu et al., 2019), life stage (Christiansen and Torrissen, 1995a, 1995b), feeding duration (Lim et al., 2019), source or type of astaxanthin (Zhang et al., 2021; Priyadarshani, 2017; White et al., 2003; Choubert and Henrich, 1993), and fish species itself (Ha et al., 1993; Schiedt et al., 1985) to have influenced astaxanthins effect on fish growth, feed utilization and survival. The functionality of vitamin E supplementation were affected by the stress status (Gao et al., 2012), feeding duration (Chen et al., 2004), dietary composition, e.g., the level of dietary lipid and HUFA (Atalah et al., 2012; Betancor et al., 2011), and the presence of antioxidants derived from other functional ingredients such as selenium and vitamin C (Chen et al., 2004). The lack of improved growth and survival in this study, irrespective of antioxidant levels, may be due to the fact that GGs were healthy and held under optimal conditions, as evidenced by the measured TAC and MDA levels. The beneficial effects of these two supplements under temperature or hypoxia conditions remain to be investigated.

5. Conclusion

The supplementation of synthetic astaxanthin at 75 mg/kg diet (0.75 g of Carophyll® Pink equivalent) was found to be sufficient to significantly improve the yellow coloration of the fins of juvenile GG after 30 days of feeding, based on the color saturation pattern. To our best knowledge this study is the first to provide a tool to modulate the color appearance of grouper, which may be used for product differentiation for the live food fish trade where the visual appearance of fish is important. Furthermore, this study highlights that astaxanthin, but not vitamin E, increased the antioxidant capacity of GG and may be a more relevant antioxidant to combat free radical formation in adverse conditions.

Notwithstanding, further studies testing lower levels of astaxanthin and vitamin E in GG under experimental stress conditions could potentially clarify the color saturation pattern and the interactive effects of these antioxidants on growth performance, coloration, and antioxidant status.

Declaration of conflicting interest

The authors do not endorse the feed supplements used in this study. We declare that there is no conflict of financial or personal interest that could have appeared to influence the work reported in this paper. The authors alone are responsible for the content and writing of the paper.

CRediT authorship contribution statement

Fernando Fernando: Conceptualization, Methodology,

Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Caroline L. Candebat:** Methodology, Formal analysis, Writing - review & editing. **Jan M. Strugnell:** Conceptualization, Methodology, Project administration, Writing - review & editing, Supervision. **Nikos Andreakis:** Methodology, Investigation. **Leo Nankervis:** Conceptualization, Methodology, Investigation, Funding acquisition, Project administration, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[CIE] Commission Internationale de l'Eclairage, 1977. Recommendations on Uniform Color Spaces, Color-difference Equations, Psychometric Color Therms. CIE, Paris.

Abdel-Hameid, N.A.H., Abidi, S.F., Khan, M.A., 2012. Dietary vitamin E requirement for maximizing the growth, conversion efficiency, biochemical composition and haematological status of fingerling Channa punctatus. Aquac. Res. 43, 226–238.

Ambati, R.R., Phang, S.M., Ravi, S., Aswathanarayana, R.G., 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. Mar. Drugs 12 (1), 128–152.

Asche, F., Bjørndal, T., Young, J.A., 2001. Market interactions for aquaculture products. Aquac. Econ. Manag. 5 (5–6), 303–318.

Atalah, E., Hernández-Cruz, C.M., Ganga, R., Ganuza, E., Benítez-Santana, T., Roo, J., Palacious, F., Izquierdo, M.S., 2012. Enhancement of gilthead seabream (*Sparus aurata*) larval growth by dietary vitamin E in relation to two different levels of essential fatty acids. Aquac. Res. 43 (12), 1816–1827.

Bell, G., McEvoy, J., Tocher, D.R., Sargent, J.R., 2000. Depletion of α-tocopherol and astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defence and fatty acid metabolism. J. Nutr. 130, 1800–1808.

Betancor, M.B., Atalah, E., Caballero, M., Benítez-Santana, T., Roo, J., Montero, D., Izquierdo, M., 2011. α-Tocopherol in weaning diets for European sea bass (*Dicentrarchus labrax*) improves survival and reduces tissue damage caused by excess dietary DHA contents. Aquac. Nutr. 17, 112–122.

Bjerkeng, B., Hamre, K., Hatlen, B., Wathne, E., 1999. Astaxanthin deposition in fillets of Atlantic salmon *Salmo salar* L. fed two dietary levels of astaxanthin in combination with three levels of α- tocopheryl acetate. Aquac. Res. 30, 637–646.

Bjerkeng, B., Hatlen, B., Jobling, M., 2000. Astaxanthin and its metabolites iodoxanthin and crustaxanthin in flesh, skin, and gonads of sexually immature and maturing Arctic charr (*Salvelinus alpinus (L.)*). Comp. Biochem. Physiol. 125B, 395–404.

Booth, M.A., Warner-Smith, R.J., Allan, G.L., Glencross, B.D., 2004. Effects of dietary astaxanthin source and light manipulation on the skin color of Australian snapper *Pagrus auratus* (Bloch and Schneider, 1801). Aquac. Res. 35, 458–464.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K., Lee, K.J., 2004. Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquaculture 242 (1–4), 553–569.

Cheng, C.H., Guo, Z.X., Ye, C.X., Wang, A.L., 2018. Effect of dietary astaxanthin on the growth performance, non-specific immunity, and antioxidant capacity of pufferfish (*Takifugu obscurus*) under high temperature stress. Fish Physiol. Biochem. 44, 209–218.

Choubert, G., 2010. Response of rainbow trout (*Oncorrhychus mykiss*) to varying dietary astaxanthin/canthaxanthin ratio: color and carotenoid retention of the muscle. Aquac. Nutr. 16, 528–535.

Choubert, G., Henrich, O., 1993. Carotenoid pigmentation of the green alga *Haematococcus pluvialis*: assay on rainbow trout *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin. Aquaculture 112, 217–226.

Christiansen, R., Torrissen, O.J., 1995b. Growth and survival of Atlantic salmon, Salmo salar L., fed different dietary levels of astaxanthin. Juveniles Aquac. Nutr. 2, 55–62.

Christiansen, R., Lie, Ø., Torrissen, O.J., 1994. Effect of astaxanthin and vitamin A on growth and survival during first feeding of Atlantic salmon, *Salmo salar* L. Aquac. Res. 25, 903–914.

Christiansen, R., Glette, J., Lie, Ø., Torrissen, O.J., Waagbø, R., 1995. Antioxidant status and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. J. Fish Dis. 18 (4), 317–328.

Christiansen, R., Lie, O., Torrissen, O.J., 1995a. Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First-feeding fry. Aquac. Nutr. 1, 189–198.

De Carvalho, C.C., Caramujo, M.J., 2017. Carotenoids in aquatic ecosystems and aquaculture: a colorful business with implications for human health. Front. Mar. Sci. 4, 93.

Del Rio, D., Stewart, A.J., Pellegrini, N., 2005. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr. Metabol. Cardiovasc. Dis. 15 (4), 316–328.

Dennis, L.P., Ashford, G., Thai, T.Q., Van In, V., Ninh, N.H., Elizur, A., 2020. Hybrid grouper in Vietnamese aquaculture: production approaches and profitability of a promising new crop. Aquaculture 522, 735108.

Doolan, B.J., Booth, M.A., Allan, G.L., Jones, P.L., 2008. Effects of dietary astaxanthin concentration and feeding period on the skin pigmentation of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801). Aquac. Res. 40 (1), 60–68.

Fang, N., Wang, C., Liu, X., Zhao, X., Liu, Y., Liu, X., Du, Y., Zhang, Z., Zhang, H., 2019. De novo synthesis of astaxanthin: From organisms to genes. Trends Food Sci. Technol. 92, 162–171.

Fanning, K.J., Paulo, C., Pun, S., Torrisi, C., Abberton, K., Exley, P., Poole, S., 2014. Astaxanthin profiles and corresponding color properties in Australian farmed black tiger prawn (*Penaeus monodon*) during frozen storage. Aquac. Res. 47, 1820–1831.

Galindo-Villegas, J., Fukada, H., Masumoto, T., Hosokaw, H., 2006. Effect of dietary immunostimulants on some innate immune responses and disease resistance against *Edwardsiella tarda* infection in Japanese flounder (*Paralichthys olivaceus*). Aquac. Sci. 54, 153–162.

Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Mamauag, R.E.P., Han, Y., 2012. Effects of dietary oxidized fish oil with vitamin E supplementation on growth performance and reduction of lipid peroxidation in tissues and blood of red sea bream Pagrus major. Aquaculture 356, 73–79.

Gomes, E., Dias, J., Silva, P., Valente, L., Empis, J., Gouveia, L., Bowen, J., Young, A., 2002. Utilization of natural and synthetic sources of carotenoids in the skin pigmentation of gilthead seabream (*Sparus aurata*). Eur. Food Res. Technol. 214 (4), 287–293.

Ha, B.S., Kang, D.S., Kim, J.H., Choi, O.S., Ryu, H.Y., 1993. Metabolism of dietary carotenoids and effects to improve the body color of cultured flounder and red sea bream. Bull. Korean Fish. Soc. 26, 91–101.

Hamre, K., 2011. Metabolism, interactions, requirements, and functions of vitamin E in fish. Aquac. Nutr. 17 (1), 98–115.

Hansen, Ø.J., Puvanendran, V., Bangera, R., 2016. Broodstock diet with water and astaxanthin improve condition and egg output of brood fish and larval survival in Atlantic cod, *Gadus morhua* L. Aquac. Res. 47 (3), 819–829.

Hart, B., Colombo, S.M., 2022. Effects of a novel weakened whole-cell form of Haematococcus pluvialis on flesh pigmentation of rainbow trout (*Oncorhynchus mykiss*) when compared to synthetic astaxanthin. Aquac. Res. 53 (6), 2408–2419.

Hunt, R.W.G., 1977. The specification of color appearance: I. Concepts and terms. Color Res. Appl. 2, 55–68.

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. J. Nat. Prod. 69, 443–449.

Kalinowski, C.T., Robaina, L.E., Fernández-Palacios, H., Schuchardt, D., Izquierdo, M.S., 2005. Effect of different carotenoid sources and their dietary levels on red porgy (*Pagrus pagrus*) growth and skin color. Aquaculture 244, 223–231.

Kalinowski, C.T., Izquierdo, M.S., Schuchardt, D., Robaina, L.E., 2007. Dietary supplementation time with shrimp shell meal on red porgy (*Pagrus pagrus*) skin color and carotenoid concentration. Aquaculture 272 (1–4), 451–457.

Kalinowski, C.T., Robaina, L.E., Izquierdo, M.S., 2011. Effect of dietary astaxanthin on the growth performance, lipid composition and post-mortem skin coloration of red porgy *Pagrus pagrus*. Aquac. Int. 19 (5), 811–823.

Kimler, V.A., Taylor, J.D., 2002. Morphological studies on the mechanisms of pigmentary organelle transport in fish xanthophores and melanophores. Microsc. Res. Tech. 58, 470–480.

Kocabas, A.M., Gatlin III, D.M., 1999. Dietary vitamin E requirement of hybrid striped bass (*Morone chrysops* female x M. saxatilis male). Aquac. Nutr. 5, 3–7.

Li, M., Chen, L., Qin, J.G., Li, E., Yu, N., Du, Z., 2013. Growth performance, antioxidant status and immune response in dark barbel catfish *Pelteobagrus vachelli* fed different PUFA/vitamin E dietary levels and exposed to high or low ammonia. Aquaculture 406, 18–27.

Lim, K.C., Yusoff, F.M., Shariff, M., Kamarudin, M.S., 2019. Dietary administration of astaxanthin improves feed utilization, growth performance and survival of Asian seabass, Lates calcarifer (Bloch, 1790). Aquac. Nutr. 25 (6), 1410–1421.

Lin, Y.H., Shiau, S.Y., 2005. Dietary vitamin E requirement of grouper, *Epinephelus malabaricus*, at two lipid levels, and their effects on immune responses. Aquaculture 248 (1–4), 235–244.
Liu, F., Qu, Y.K., Wang, A.M., Yu, Y.B., Yang, W.P., Lv, F., Nie, Q., 2019. Effects of carotenoids on the growth performance, biochemical parameters, immune responses, and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*) under high temperature stress. Aquaculture 503, 293–303.
Liu, J., Sun, Z., Gerken, H., Liu, Z., Jiang, Y., Chen, F., 2014. *Chlorella zofingiensis* as an alternative microalgal producer of astaxanthin: biology and industrial potential. Mar. Drugs 12, 3487–3515.
Long, L., Zhang, H., Ni, Q., Liu, H., Wu, F., Wang, X., 2019. Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 219, 25–34.
Lorenz, R.T., Cysewski, G.R., 2000. Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. Trends Biotechnol. 18 (4), 160–167.
Lozano, A.R., Borges, P., Robaina, L., Betancor, M., Hernandez-Cruz, C.M., García, J.R., Caballero, M.J., Vergara, J.M., Izquierdo, M., 2017. Effect of different dietary vitamin E levels on growth, fish composition, fillet quality and liver histology of meagre (*Argyrosomus regius*). Aquaculture 468, 175–183.
Luo, K., Li, J., Chen, J., Pan, Y., Zhang, Y., Zhou, H., Zhang, W., Mai, K., 2020. Proteomics analysis of skin coloration of large yellow croaker *Larimichthys crocea* fed different dietary carotenoids. Aquac. Nutr. 26 (6), 1981–1993.
Machlin, L.J., Bendich, A., 1987. Free radical tissue damage: protective role of antioxidant nutrients. FASEB J. 1, 441–445.
Miki, W., Yamaguchi, K., Konosu, S., Takane, T., Satake, M., Fujita, T., Kuwabara, H., Shimeno, S., Takeda, M., 1985. Origin of tunaxanthin in the integument of yellowtail (Seriola quinqueradiata). Comp. Biochem. Physiol. 80B, 195–201.
Montero, D., Tort, L., Robaina, L., Vergara, J.M., Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish Shellfish Immunol. 11 (6), 473–490.
Naguib, Y.M., 2000. Antioxidant activities of astaxanthin and related carotenoids. J. Agric. Food Chem. 48 (4), 1150–1154.
Nakano, T., Tosa, M., Takeuchi, M., 1995. Improvement of biochemical features in fish health by red yeast and synthetic astaxanthin. J. Agric. Food Chem. 43 (6), 1570–1573.
Nankervis, L., Cobcroft, J.M., Nguyen, N.V., Rimmer, M.A., 2021. Advances in practical feed formulation and adoption for hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂*) aquaculture*. Rev. Aquac.
Niu, J., Wen, H., Li, C.H., Liu, Y.J., Tian, L.X., Chen, X., Huang, Z., Lin, H.Z., 2014. Comparison effect of dietary astaxanthin and β-carotene in the presence and absence of cholesterol supplementation on growth performance, antioxidant capacity and gene expression of *Penaeus monodon* under normoxia and hypoxia condition. Aquaculture 422–423, 8–17.
Nocillado, J., Elizur, A., Palma, P., Dennis, L., Ninh, N.H., Jesus-Ayson, E.G., In, V.V., Thai, T.Q., Anderson, K., Luu, T.H.G., Knibb, W., Knuckey, R., Canépa, M., Brigh, D., 2021. Developing Technologies for Giant Grouper (Epinephelus lanceolatus) Aquaculture in Vietnam, the Philippines and Australia. Australian Centre for International Agricultural Research (ACIAR). ISBN 978-1-922635-24-2.
Olsen, R.E., Baker, R.T.M., 2006. Lutein does not influence flesh astaxanthin pigmentation in the Atlantic salmon (*Salmo salar* L.). Aquaculture 258 (1–4), 558–564.
Peng, L.I., Gatlin III, D.M., 2009. Dietary vitamin E requirement of the red drum *Sciaenops ocellatus*. Aquac. Nutr. 15, 313–319.
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.
Pozo, R., Lavety, J., Love, R.M., 1988. The role of dietary α-tocopherol (vitamin E) in stabilizing the canthaxanthin and lipids of rainbow trout muscle. Aquaculture 73, 165–175.
Priyadarshani, A.M.B., 2017. A review on factors influencing bioaccessibility and bioefficacy of carotenoids. Crit. Rev. Food Sci. Nutr. 57, 1710–1717.
Puangkaew, J., Kiron, V., Somamoto, T., Okamoto, N., Satoh, S., Takeuchi, T., Watanabe, T., 2004. Non-specific immune response of rainbow trout (*Oncorhynchus mykiss* Walbaum) in relation to different status of vitamin E and highly unsaturated fatty acids. Fish Shellfish Immunol. 16 (1), 25–39.
Rimmer, M.A., Glamuzina, B., 2019. A review of grouper (Family Serranidae: Subfamily Epinephelinae) aquaculture from a sustainability science perspective. Rev. Aquac. 11, 58–87.
Safari, O., Atashi, M.M.S., 2015. The effects of dietary supplement of annatto (*Bixa orellana*) seed meal on blood carotenoid content and fillet color stability in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 937, 275–281.
Sahoo, P.K., Mukherjee, S.C., 2002. Influence of high dietary α-tocopherol intakes on specific immune response, non-specific resistance factors and disease resistance of healthy and aflatoxin B1-induced immunocompromised Indian major carp, *Labeo rohita* (Hamilton). Aquac. Nutr. 8 (3), 159–167.
Schiedt, K., Leuenberger, F.J., Vecchi, M., Glinz, E., 1985. Absorption retention and metabolic transformations of carotenoids in rainbow trout salmon and chicken. Pure Appl. Chem. 57, 685–692.
Sefc, K.M., Brown, A.C., Clotfelter, E.D., 2014. Carotenoid-based coloration in cichlid fishes. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 173, 42–51.
Segner, H., Arend, P., Von Poeppinghausen, K., Schmidt, H., 1989. The effect of feeding astaxanthin to Oreochromis niloticus and Colisa labiosa on the histology of the liver. Aquaculture 381–390.
Sun, X., Chang, Y., Ye, Y., Ma, Z., Liang, Y., Li, T., Jiang, N., Xing, W., Luo, L., 2012. The effect of dietary pigments on the coloration of Japanese ornamental carp (koi, *Cyprinus carpio* L.). Aquaculture 342–343, 62–68.
Sung, W.C., Lin, J.R., Chiou, T.K., Chang, Y.W., 2019. Selected quality attributes of frozen farmed giant grouper (*Epinephelus lanceolatus*) as a function of storage temperature. J. Mar. Sci. Technol. 27 (3), 267–275.
Tejera, N., Cejas, J.R., Rodríguez, C., Bjerkeng, B., Jerez, S., Bolaños, A., Lorenzo, A., 2007. Pigmentation, carotenoids, lipid peroxides and lipid composition of skin of red porgy (*Pagrus pagrus*) fed diets supplemented with different astaxanthin sources. Aquaculture 270 (1–4), 218–230.
Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz, E., Bell, J.G., Geurden, I., Lavens, P., Olsen, Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquac. Nutr. 8 (3), 195–207.
Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz, E., Wille, M., Bell, J. G., Olsen, Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidized oil and vitamin E. Aquac. Int. 11 (1–2), 195–216.
Van Belleghem, S.M., Papa, R., Ortiz-Zuazaga, H., Hendrickx, F., Jiggins, C.D., Owen McMillan, W., Counterman, B.A., 2018. patternize: an R package for quantifying color pattern variation. Methods Ecol. Evol. 9, 390–398.
Viera, I., Perez-Galvez, A., Roca, M., 2018. Bioaccessibility of marine carotenoids. Mar. Drugs 16 (10), 397.
Wade, N.M., Gabaudan, J., Glencross, B.D., 2017. A review of carotenoid utilization and function in crustacean aquaculture. Rev. Aquac. 9 (2), 141–156.
Wang, Y.J., Chien, Y.H., Pan, C.H., 2006. Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins, *Hyphessobrycon callistus*. Aquaculture 261 (2), 641–648.
Weller, H.I., Westneat, M.W., 2019. Quantitative color profiling of digital images with earth mover's distance using the R package color distance. PeerJ 7, e6398.
White, D.A., Moody, A.J., Serwata, R.D., Bowen, J., Soutar, C., Young, A.J., Davies, S.J., 2003. The degree of carotenoid esterification influenced the absorption of astaxanthin in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Nutr. 9, 247–251.
Yi, X., Xu, W., Zhou, H., Zhang, Y., Luo, Y., Zhang, W., Mai, K., 2014. Effects of dietary astaxanthin and xanthophylls on the growth and skin pigmentation of large yellow croaker *Larimichthys croceus*. Aquaculture 433, 377–383.
Zhang, C., Jin, Y., Yu, Y., Xiang, J., Li, F., 2021. Effects of natural astaxanthin from microalgae and chemically synthetic astaxanthin supplementation on two different varieties of the ridgetail white prawn (*Exopalaemon carinicauda*). Algal Res. 57, 102347.
Zhou, Q.C., Wang, L.G., Wang, H.L., Wang, T., Elmada, C.Z., Xie, F.J., 2013. Dietary vitamin E could improve growth performance, lipid peroxidation and non-specific immune responses for juvenile cobia (*Rachycentron canadum*). Aquac. Nutr. 19, 421–429.
Zhu, X., Hao, R., Zhang, J., Tian, C., Hong, Y., Zhu, C., Li, G., 2022. Dietary astaxanthin improves the antioxidant capacity, immunity and disease resistance of coral trout (*Plectropomus leopardus*). Fish Shellfish Immunol. 122, 38–47.