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Research Article

Dietary DHA Oil Supplementation Promotes Ovarian Development and Astaxanthin Deposition during the Ovarian Maturation of Chinese Mitten Crab *Eriocheir sinensis*

Xiaodong Jiang,[1,2] **Kewu Pan**,[1] **Yuhong Yang**,[2] **Alexander Chong Shu-Chien**,[3,4] **and Xugan Wu**[1,5,6]

[1]*Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture and Rural Affairs, Shanghai Ocean University, Shanghai 201306, China*
[2]*College of Animal Science and Technology, Northeast Agricultural University, Harbin 150036, China*
[3]*School of Biological Sciences, Universiti Sains Malaysia, Minden, 11800 Penang, Malaysia*
[4]*Center for Chemical Biology, Universiti Sains Malaysia, Sains@USM, Blok B No. 10, Persiaran Bukit Jambul, 11900 Bayan Lepas, Penang, Malaysia*
[5]*National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai 201306, China*
[6]*Shanghai Collaborative Innovation for Aquatic Animal Genetics and Breeding, Shanghai Ocean University, Shanghai 201306, China*

Correspondence should be addressed to Xugan Wu; xgwu@shou.edu.cn

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Astaxanthin (Axn) is an essential carotenoid for crustacean pigmentation, and docosahexaenoic acid (DHA) is an important fatty acid; both play key roles in maintaining the health of many aquaculture species. The present study explored the combined effect of dietary Axn and DHA on gonadal development and carotenoid deposition in adult females of the Chinese mitten crab, *Eriocheir sinensis*. A 2 × 3 factorial design of experimental diets was created to contain two levels of Axn (0 mg/kg and 100 mg/kg) and three levels of DHA oil (0%, 0.33%, and 0.67%). The results showed as follows: (1) For the culture performance, dietary DHA oil significantly increased the gonadosomatic index (GSI), and Diet 2 (Axn 0% + DHA oil 0.33%) had the highest GSI among all treatments. (2) For the enzymatic indicators in the hepatopancreas and hemolymph, supplementation with 0.33% DHA oil significantly improved the antioxidant capacity (T-AOC and MDA), immunity (AKP and ACP), and health status (e.g., GPT and GOT) of *E. sinensis*. (3) Supplementation with 100 mg/kg Axn significantly increased redness (a∗) and Axn concentration in both the ovaries and hepatopancreas, and supplementation with 0.33% or 0.67% DHA oil produced a further significant improvement in Axn concentration when the diets were supplemented with 100 mg/kg of Axn. (4) As for proximate composition, dietary Axn and DHA significantly increased the deposition of total lipids and triacylglycerol in the hepatopancreas. As expected, the crabs fed diets with DHA supplementation showed an increase in the DHA percentage and DHA/EPA ratio in the ovaries and hepatopancreas. In conclusion, dietary Axn and DHA oil had positive effects on ovarian development in *E. sinensis* females. The optimal combination of dietary Axn and DHA oil was determined to be approximately 100 mg/kg and 0.33%, respectively, for this species during ovarian maturation.

1. Introduction

Carotenoids are important pigments and antioxidants produced naturally by plants, bacteria, and fungi [1, 2]. Although more than 600 carotenoids have been found in nature, only a few of them can be absorbed and stored in animal tissues, among which astaxanthin (Axn) is the most well-known and well-studied carotenoid [3, 4]. Axn is one

of the important carotenoids, which widely exists in plankton, arthropods, and fish species, and its positive effects on colouration have been reported in many crustacean species [5–8]. In addition to pigmentation properties, extensive studies have shown many other benefits of Axn in crustaceans, including enhanced growth performance, gonadal development, reproductive performance, antioxidant activity, immunity enhancement, and environmental stress and disease resistance [9, 10]. Although Axn is an effective carotenoid that is widely used for crustacean pigmentation, it is extremely expensive and undesirably adds to the total cost of commercial feeds [11, 12]. Moreover, even though the apparent digestibility coefficient (ADC) values for Axn in crustaceans are generally high (about 65.3-98.5%), the retention efficiencies of Axn in crustacean are considerably low, being in the range of only 14.3-26.9% for *Penaeus monodon* [13, 14]. Therefore, deeper levels of comprehension on absorption and deposition of Axn in crustacean animals could improve its utilisation efficiency and reduce production costs, which have become important issues in crustacean farming. As a lipid-soluble compound, the bioavailability and absorption of Axn may be influenced by the amount and type of dietary lipids, as reported with *Lysmata vannamei* [15] and *P. monodon* [13, 16].

Docosahexaenoic acid (22:6n-3, DHA) is one of the key omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) required for crustacean species. Previous studies have reported the beneficial effects of dietary DHA on growth performance, SOD activity, and hypoxia stress resistance in crustacean species [17, 18]. Marine fish oil is a common source of DHA and EPA for aquafeed, but global production is unable to meet the fast-growing demands of the aquafeed industry [19]. Therefore, the search for sustainable and cost-effective alternatives to fish oil is crucial [20]. In recent decades, some GM oilseed and microorganism (e.g., microalgae and bacteria) products have been considered as promising new sources of DHA and EPA [19]. Among them, the microalgae *Schizochytrium* sp., a fast-growing thraustochytrid with as much as 50% DHA in the extracted lipids [21, 22], and the DHA oil/DHA-rich meal of *Schizochytrium* sp. are already commercially available products [23, 24]. Previous research had reported the potential of *Schizochytrium* sp. products as dietary DHA supplements in shrimp farming [24–28].

The Chinese mitten crab *Eriocheir sinensis* is an economically important freshwater crab in China and is popular in the Chinese market due to its high nutritional quality and delicious flavour [29, 30]. Since the 1990s, the farming industry for *E. sinensis* has experienced rapid development after the breakthrough of commercial-scale hatchery technology [31]. Although important progress had been made in the cultural techniques of *E. sinensis* during the past 20 years, the quality of eggs and larvae produced by pond-cultured *E. sinensis* broodstock is highly variable and remains a major constraint for hatchery production of this species [32, 33]. Nutrition status is known to be vital for the reproductive performance of crustacean broodstock; thus, a more comprehensive knowledge on nutritional requirements of *E. sinensis* broodstock could contribute to the improvement of larval quality and cost-effective hatchery production [32–34]. Despite the importance of Axn [7, 35] and LC-PUFAs [33, 36, 37] for ovarian development of *E. sinensis* had been demonstrated in previous studies, their combined effects on *E. sinensis* remain unclear. Moreover, the combined effects of dietary Axn and LC-PUFAs on crustaceans have been demonstrated in several species, including the hermaphroditic shrimp *Lysmata wurdemanni* [38], *L. vannamei* [39], and the giant tiger prawn *P. monodon* [40]. Considering that DHA is one of the key n-3 LC-PUFAs required for crustacean species as inspired by previous studies, this study was conducted to investigate the combined effect of dietary Axn and DHA on adult female *E. sinensis* regarding ovarian development, antioxidant capacity, colour parameters, carotenoid concentration, and biochemical composition during the ovarian maturation stage. These results are expected to lead to a better estimation of optimal levels of Axn and DHA supplementation on gonad development in *E. sinensis*, as well as potentially reveal the possible interactive functions of Axn and DHA, which may not be expressed in a single factor study.

2. Materials and Methods

2.1. Diet Formulation and Preparation. As shown in Table 1, six experimental diets were formulated to contain two levels of Axn supplementation (0 mg/kg and 100 mg/kg) and three levels of DHA oil supplementation (0%, 0.33%, and 0.67%) in a factorial design (2 × 3). The supplementation level of dietary Axn and DHA oil in experimental diets were determined based on published studies that have evaluated the optimal levels of these two nutrients in *E. sinensis* diets [18, 41].

Synthetic Axn (purity = 10%) donated by DSM (China) Co., Ltd. (Shanghai, China) was used as the Axn source, and the DHA oil extracted from marine algae *Schizochytrium limacinum* (containing 45% DHA) was provided by Qingdao Keyuan Marine Biochemistry Co., Ltd. (Qingdao, China). Dry feed ingredients were smashed to pass a 180 μm sieve using a grinder (WF-30B, Jiangyin Dachuang Mechanical Manufacture Co., Ltd., Jiangsu Province, China). All dry ingredients were thoroughly blended with a commercial mixer (XHS-50, Ningbo Beilun Thermal Machinery Manufacturing Co., Ltd., Zhejiang Province, China). The lipid ingredients were then blended in slowly until a homogenous mixture was obtained and water was added to form a moist dough. Mixtures were then extruded into sinking pellets (3.5 mm in diameter) by a single-screw extruder (DSE30, Jinan Dingrun Machinery Co., Ltd., Shandong Province, China). The pellets were dried at room temperature using a dehumidifier (GE DRY C20 C, General Electric Co., Ltd., USA) for 48 h. To avoid the loss of Axn and DHA in feeds, the experimental diets were stored at -20°C until use. The formulation of experimental diets is shown in Table 1, and the proximate composition and carotenoid content of experimental diets are shown in Supplementary Table 1.

2.2. Experimental Design and Feeding Trial. The culture experiment was run in 18 polyethylene (PE) tanks (diameter × height = 108 × 120 cm) with a recycling system

Table 1: Formulations of the experimental diets (%).

Items	Diet-1 Axn 0 + DHA oil 0	Diet-2 Axn 0 + DHA oil 0.33	Diet-3 Axn 0 + DHA oil 0.67	Diet-4 Axn 100+ DHA oil 0	Diet-5 Axn 100+ DHA oil 0.33	Diet-6 Axn 100+ DHA oil 0.67
Soybean meal	20.00	20.00	20.00	20.00	20.00	20.00
Rapeseed meal	8.00	8.00	8.00	8.00	8.00	8.00
Peanut meal	8.00	8.00	8.00	8.00	8.00	8.00
Fish meal	22.00	22.00	22.00	22.00	22.00	22.00
Fish soluble	8.00	8.00	8.00	8.00	8.00	8.00
Brewer yeast	2.00	2.00	2.00	2.00	2.00	2.00
Wheat flour	15.55	15.55	15.55	15.55	15.55	15.55
CMC	3.00	3.00	3.00	3.00	3.00	3.00
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil	4.00	4.00	4.00	4.00	4.00	4.00
Rapeseed oil	1.50	1.50	1.50	1.50	1.50	1.50
Soybean oil	1.50	1.50	1.50	1.50	1.50	1.50
Cholesterol	0.40	0.40	0.40	0.40	0.40	0.40
Choline chloride	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin premix[a]	0.40	0.40	0.40	0.40	0.40	0.40
Mineral premix[b]	0.25	0.25	0.25	0.25	0.25	0.25
$Ca(H_2PO_4)_2$	1.20	1.20	1.20	1.20	1.20	1.20
Taurine	0.30	0.30	0.30	0.30	0.30	0.30
Cellulose	0.50	0.50	0.50	0.38	0.38	0.38
Glycerol trioleate	1.00	0.67	0.33	1.00	0.67	0.33
Astaxanthin (10%)[c]	0.00	0.00	0.00	0.12	0.12	0.12
DHA oil (45%)[d]	0.00	0.33	0.67	0.00	0.33	0.67

[a]Vitamin premix (per kg diet): vitamin A, 13125 IU; vitamin D_3, 5250 IU; vitamin E, 140 IU; vitamin K_3, 14 mg; vitamin B_1, 21 mg; vitamin B_2, 33.6 mg; vitamin B_6, 28.7 mg; vitamin B_{12}, 0.07 mg; biotin, 0.28 mg; D-calcium pantothenate, 77 mg; folic acid, 7 mg; nicotinamide, 175 mg; vitamin C, 490 mg; inositol, 595 mg; ethoxyquin, 1.75 mg. [b]Mineral premix (per kg diet): $FeSO_4$, 45 mg; $ZnSO_4$, 215 mg; $CuSO_4$, 90 mg; $MnSO_4$, 35 mg; Na_2SeO_3, 1 mg; $CoSO_4$, 5 mg; Ca $(IO_3)_2$, 1.5 mg. [c]Synthetic astaxanthin, provided by DSM (China) Co., Ltd., Shanghai, China. [d]Provided by Qingdao Keyuan Marine Biochemistry Co., Ltd., Qingdao, China.

at the Chongming Research Station of Shanghai Ocean University. In late August 2019, 400 adult females were randomly selected from an earthen pond at the station and transported to indoor PE tanks for acclimation. The initial body weight of experimental crabs was set within the range of 70 g to 90 g, which is the typical weight of adult female crabs that have recently completed puberty moulting. The maturation of adult crabs was determined based on their secondary sex characteristics as descript by Wu et al. [42]. After 5 days of domestication in the tank, lively and whole crabs were selected for subsequent culture. Each dietary treatment had triplicate tanks with a density of 16 females per tank, which almost approached the highest density at which the normal growth of adult crabs may be guaranteed. Artificial water plants (30 cm tall) were set at the bottom of the tanks as crab shelters, and the coverage of artificial plants was set at approximately 50% in each tank. The experimental water maintained a fixed level of 50 cm in each tank. Filtered water was supplied to the tanks at a flow rate of 3 L/min, and all tanks were aerated continuously with air stones to maintain a 5 mg/mL higher dissolved oxygen (DO) concentration. Fluorescent lamps provided light with a photoperiod of 12 L/12 D. During the culture trial, the crabs were fed daily at a rate of 3% of their body weight, and all tanks were siphoned daily to remove excreta and uneaten diets. Throughout the experiment, the pH and ammonia-N and nitrite concentrations of the water were 7.5-8.2, 0.2-0.4 $mg{\cdot}L^{-1}$, and 0.05-0.15 $mg{\cdot}L^{-1}$, respectively, which are suitable for the growth of adult *E. sinensis* [43]. The experiment lasted for 70 days and finished on November 10, 2019.

2.3. Growth Data and Sample Collection. On day 35 of the feeding test, three females were randomly selected from each tank after 24 h of fasting, and their weights were measured

with a digital balance (precision = 0.01 g, JY202, Shanghai Puchun Measure Instrument Co., Ltd., Shanghai, China). Thereafter, the ovaries and hepatopancreas were dissected with 10 cm ophthalmic forceps and 10 cm surgical scissors, and the dissected tissues were weighed to calculate gonadosomatic index (GSI) and hepatosomatic index (HSI). Similarly, on day 70 of feeding test, three crabs from each tank were randomly selected to measure their body weights. Approximately, 2 mL of hemolymph was collected from the base of the crab's third walking limb by inserting a syringe needle (maximum capacity 1 mL), and the collected hemolymph was then stored in 2 mL tubes at -80°C until use. The crabs were then dissected to get the ovaries and hepatopancreas, while the muscles in the rest of the body were carefully removed and weighed. The carapace, muscles, hepatopancreas, and ovaries of each crab were stored at -40°C for subsequent biochemical analyses and measurements. The weight gain rate (WGR), GSI, HSI, and survival were calculated with the following formulae:

$$\begin{aligned}
WGR\,(\%) &= 100 \times \frac{(\text{Final individual weight} - \text{Initial individual weight})}{\text{Initial individual weight}}.\\
GSI\,(\%) &= 100 \times \frac{\text{Gonad wet weight}}{\text{Body wet weight}}.\\
HSI\,(\%) &= 100 \times \frac{\text{Hepatopancreas wet weight}}{\text{Body wet weight}}.\\
\text{Survival}\,(\%) &= 100 \times \frac{\text{final crab number}}{(\text{initial crab number} - \text{the number of crabs sampled on day 35})}.
\end{aligned} \tag{1}$$

2.4. Antioxidant Capacity and Non-specific Immunity. The thawed hemolymph samples were homogenised with a IKA micro homogeniser (T10B, IKA Co., Germany) and were centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was collected and stored at -40°C in a refrigerator for the next measurement. About 0.1 g of hepatopancreas from each crab was weighed and added to the saline solution at a ratio of 1 : 5 (*w*/*v*) and then were homogenised with an IKA homogeniser in a 2 mL centrifuge tube. Homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and stored at -40°C for next analysis. The same volume of supernatant phases from each sample from the same replicate tank was pooled and mixed before subsequent analysis.

The levels of total antioxidant capacity (T-AOC, No. A015-2-1), glutathione peroxidase (GSH-Px, No. A005-1-2), malondialdehyde (MDA, No. A003-1-1), superoxide dismutase (SOD, No. A001-1-2), catalase (CAT, No. A015-2-1), alkaline phosphatase (AKP, No. A059-1-1), acid phosphatase (ACP, No. A060-1), glutamic pyruvic transaminase (GPT, No. C009-1), and glutamic oxalacetic transaminase (GOT, No. C010-1) in the hepatopancreas and hemolymph were analysed with a spectrophotometer and corresponding detection kits (Nanjing Jiancheng Biological Product, China). The levels of T-AOC, GSH-Px, MDA, SOD, CAT, AKP, ACP, GPT, and GOT were analysed according to the manufacturer's guidelines, and 50, 50, 50, 40, 25, 50, 15, 15, 20, and 20 μL of supernatant were used for each determination with the wavelengths of 520, 412, 410, 532, 550, 520, 520, 520, 505, and 505 nm, respectively.

2.5. Carotenoids and Colour Parameters. The ovaries, hepatopancreas, and carapaces of each crab were freeze-dried and ground separately before carotenoid extraction, and the same tissues of three crabs in each test tank were pooled as the composite sample for extraction. The total carotenoids in all samples were extracted with acetone and determined by the ultraviolet-visible spectrophotometer (T6 New Century, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) at 470 nm wavelength [44]. The esterified carotenoids were first hydrolysed by enzymatic hydrolysis [45], and then, the Axn, lutein, zeaxanthin, canthaxanthin, and β-carotene were analysed by an Agilent high-performance liquid chromatograph (HPLC) using an Agilent 1260 HPLC system (Agilent Technologies Inc., CA, USA). The HPLC system was equipped with a YMC Carotenoid C30 column (4.6 × 150 mm, diameter of packing = 3 μm, YMC Co., Ltd., Kyoto, Japan). The gradient mobile phases of the Agilent 1260 HPLC system consisted of A and B, in which mobile phase A consisted of methyl alcohol : methyl tert-butyl ether : formic acid (3 : 2 : 0.01, *v*/*v*/*v*), and mobile phase B was a mixture of alcohol : triethylamine (100 : 0.04, *v*/*v*). The five carotenoids were identified and quantified based on commercially available standards, and the detailed steps of carotenoid analysis used in this experiment were described in Long et al. [7].

The ovaries, hepatopancreas, and carapace of experimental crabs were freeze-dried and ground separately before colour parameter measurement. The colour values of samples were quantified in CIE2000 L∗a∗b∗ colour space by a colorimeter (CR400, Konica Minolta, Tokyo, Japan) and the values of L∗, a∗, and b∗ representing lightness, redness, and yellowness, respectively [46]. The colour parameters were measured in nine carbs from each feed treatment (3 crabs/replicate). Six relatively smooth points were selected on the tissue surface to measure colour values, and the results were expressed as the average value for each crab.

2.6. Biochemical Analysis. The same crab tissues from each experimental tank were pooled as a composite sample before the biochemical analysis. The analyses of moisture (No. 950-46), crude protein (No. 2001-11), and ash (No. 920-153) in the muscle, hepatopancreas, and ovaries were performed according to the Association of Official Analytical Chemists (AOAC) procedures [47]. The total lipids were extracted with chloroform : methanol (2 : 1, *v*/*v*) according to Folch et al. [48].

The lipid classes were quantified using an Iatroscan MK-6s TLC-FID analyser (Iatron Laboratories Inc., Tokyo, Japan) according to the method described by Wu et al. [49]. The developing solvent system used was hexane : diethyl ether : formic acid (42 : 28 : 0.3, *v*/*v*/*v*). Thereafter, lipid classes were detected with a hydrogen flame ionisation detector (FID) under the conditions of hydrogen 160 mL/min and air 2 L/min, and the thin-layer chromatogram was processed with the Chormstar software. Lipid classes in crab tissues were quantified as total phospholipids (PL, including phosphatidylcholine phosphatidylethanolamine phosphatidylinositol and other phospholipids), triacylglycerol (TG), free fatty acids (FFA), and cholesterol (CHO). The

level of each lipid class was expressed as milligrams of lipid class per gram of wet tissue (mg/g).

For the fatty acid analysis, the total lipid concentration of the samples was esterified by boiling 14% boron trifluoride/methanol (*w*/*w*), and the fatty acid methyl esters (FAMEs) were extracted with hexane [33]. The FAMEs were analysed using an Agilent 7890B GC/5977A gas chromatograph-mass spectrometer (GC-MS) with an Omegawax-320 fused silica capillary column (30 m × 0.32 mm × 0.25 μm, Supelco, Bellefonte, PA, USA). The injector and detector temperatures were maintained at 260°C. The column temperature was initially set at 40°C, then increased at a rate of 10°C min^{-1} to 170°C and held for 1 min, then further increased at a rate of 2°C min^{-1} to 220°C, which would hold 1 min. It was ulteriorly increased at a rate of 2°C min^{-1} to the final temperature of 230°C and held for 30 min until all FAMEs had been eluted. The peaks were determined by comparing retention times with Supelco-37 component FAME standard mixture (CRM47885, Sigma-Aldrich Co., St. Louis, MO, USA), and individual fatty acids were quantified with reference to internal standard. Fatty acid composition was expressed as the percentage of each fatty acid in total fatty acid content.

2.7. Statistical Analysis. The SPSS 26.0 software was used for statistical analyses. All data were expressed as means with pooled standard deviation (SD) values, and homogeneity of data variance was analysed using Levene's test. One-way ANOVA was used to statistical analysis of all experimental data, and Tukey's multiple range test was used to determine differences. When homogeneity of variances was not achieved, the data were test using the Kruskal–Wallis *H* nonparametric test, followed by the Games–Howell nonparametric multiple comparison test. The interactive effects of dietary Axn and DHA on experimental data of adult female crab (*E. sinensis*) were studied by two-way variance analysis. For all statistical analyses, the samples from each tank were treated as one experimental unit, and the data of all indices for each tank (replicate) were considered as the mean values for statistical analyses. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Gonadal Development and Proximate Composition. Table 2 shows the WGR, GSI, and HSI for the six dietary treatments. At the first sampling point (day 35), no individual or interactive effect of Axn or DHA on gonad development of adult *E. sinensis* was observed. At the second sampling point (day 70), 0.33% DHA oil (Diets 2 and 5) caused significant increases in *E. sinensis* GSI, with the highest value ($P < 0.05$) obtained in Diet 2 treatment. The WGR, HSI, and final survival did not show any significant difference among treatments at day 70.

Table 3 shows the proximate composition of crabs fed the six diets. Significant differences within ovaries were detectable only in the ash content, which was significantly higher in Diet 3 (Axn 0 + DHA oil 0.67%) than in Diets 1, 4, and 5. The most significant differences were observed in the hepatopancreas, in which lipid contents were significantly lower but moisture contents were significantly higher in the control (Diet 1) treatment. Moreover, 100 mg/kg Axn and 0.33% DHA oil (Diet 5) significantly increased the ash content in the hepatopancreas ($P < 0.05$). Dietary Axn and DHA had a slight effect on the proximate composition of *E. sinensis* muscle ($P > 0.05$).

3.2. Antioxidant Capacity and Non-specific Immune Indices. Table 4 presents the antioxidant indices of adult female *E. sinensis*. Supplementation with 100 mg/kg Axn (Diets 4-6) significantly increased T-AOC levels in both hepatopancreas and hemolymph ($P < 0.05$). Dietary supplementation with 0.33% DHA oil (Diets 2 and 5) significantly increased hepatosomatic T-AOC levels, but T-AOC levels significantly decreased with the further increase of DHA oil supplementation from 0.33% to 0.67%. On the contrary, crabs fed diets supplemented with 0.33% DHA oil had significantly lower MDA levels in the hemolymph, whereas higher DHA oil levels (0.67%) dramatically increased MDA levels in the hemolymph ($P < 0.05$). Dietary Axn and DHA, as well as their interaction, had no significant effect on the SOD activities in both hepatopancreas and hemolymph.

Table 5 presents the nonspecific immune indices and physiological status of *E. sinensis* fed different diets. In the three Axn-control treatments (Diets 1-3), hepatosomatic GPT and GOT levels showed a "high-low-high" trend with dietary DHA supplementation only, but they showed a decreasing trend with dietary DHA supplementation in three Axn-supplemented diets (Diets 4-6). Supplementation with 100 mg/kg Axn significantly increased ACP activities but significantly decreased GPT and GOT activities in the hemolymph ($P < 0.05$). Significant interactions between Axn and DHA were found on ACP, AKP, and GPT levels in the hemolymph.

3.3. Colour Parameters and Carotenoid Composition. Figures 1–3 show the colour parameters of *E. sinensis* individuals who were fed six experimental diets. After 70 days of feeding, 100 mg/kg Axn significantly decreased the lightness (L∗) and brightness (b∗) of the ovaries but significantly increased their redness (a∗) ($P < 0.05$). Regarding the influence of DHA on ovarian colour, the redness (a∗) of the ovaries showed an increasing trend with DHA oil supplementation from 0% to 0.67% with no significant difference. Supplementation with 100 mg/kg Axn also significantly increased the redness (a∗) of the hepatopancreas, whereas 0.67% DHA oil significantly increased the lightness (L∗) and brightness (b∗) of the hepatopancreas in Axn-control treatments ($P < 0.05$). Colour parameters of the carapace showed no significant differences among treatments (Figure 3, $P > 0.05$).

The carotenoid content of *E. sinensis* is shown in Table 6. The contents of total carotenoid, Axn, and zeaxanthin in the ovaries markedly increased after feeding diets containing 100 mg/kg Axn ($P < 0.05$). The effect of dietary DHA was also noted for Axn deposition in the ovaries, while the Axn and total carotenoid contents showed an increasing trend with dietary DHA supplementation. In the hepatopancreas, the contents of total carotenoid, Axn, and zeaxanthin were

Table 2: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on weight gain rate (WGR), gonadosomatic index (GSI), hepatosomatic index (HSI), and survival of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
0 day										
Body weight (g)	74.33	76.59	75.55	76.43	74.29	75.81	0.89	0.383	0.504	0.361
35 days										
Body weight (g)	78.53	81.07	79.46	80.95	78.47	80.21	0.97	0.502	0.455	0.576
WGR (%)	5.66	5.85	5.18	5.91	5.62	5.81	1.10	0.577	0.388	0.221
GSI (%)	6.69	6.56	6.74	6.74	7.30	7.66	0.68	0.173	0.617	0.502
HSI (%)	7.95	8.01	8.45	7.49	7.95	7.87	0.61	0.286	0.339	0.440
70 days										
Body weight (g)	78.33	80.61	79.41	80.73	78.44	80.33	0.66	0.354	0.077	0.229
WGR (%)	5.39	5.25	5.11	5.63	5.59	5.96	1.32	0.721	0.557	0.703
GSI (%)	8.24^{a}	10.25^{b}	9.08^{ab}	9.44^{ab}	9.93^{b}	9.33^{ab}	0.84	0.316	0.040	0.212
HSI (%)	5.26	5.73	5.60	5.23	5.78	5.11	0.48	0.392	0.102	0.233
Survival (%)	75.56	84.44	82.22	80.00	84.44	82.22	8.55	0.950	0.235	0.593

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). WGR: weight gain rate; GSI: gonadosomatic index; HSI: hepatopancreas index.

Table 3: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on proximate composition (% wet weight) of ovaries, hepatopancreas, and muscle of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
Ovaries										
Moisture	52.63	50.66	50.03	50.75	50.50	51.74	1.40	0.396	0.665	0.115
Protein	29.81	30.70	30.87	30.94	31.46	30.39	1.07	0.452	0.434	0.600
Lipid	15.57	16.38	16.84	16.36	15.72	15.45	0.68	0.208	0.155	0.006
Ash	2.66^{a}	3.55^{ab}	4.44^{b}	2.36^{a}	2.41^{a}	3.66^{ab}	0.48	0.442	0.224	0.002
Hepatopancreas										
Moisture	76.25^{b}	65.56^{a}	69.72^{a}	68.07^{a}	66.12^{a}	69.34^{a}	3.37	0.634	0.037	0.031
Protein	11.68	11.80	12.49	11.18	10.30	10.48	1.30	0.478	0.248	0.382
Lipid	6.50^{a}	12.80^{b}	12.98^{b}	11.69^{b}	11.95^{b}	11.65^{b}	2.74	0.529	0.435	0.024
Ash	2.30^{a}	3.04^{ab}	2.74^{ab}	3.34^{ab}	3.46^{b}	2.64^{ab}	0.39	0.788	0.012	0.225
Muscle										
Moisture	80.79	80.04	79.27	80.72	80.11	81.03	1.36	0.442	0.693	0.129
Protein	15.87	15.13	16.69	15.61	16.86	16.16	1.01	0.063	0.523	0.946
Lipid	1.04	1.04	1.08	1.07	1.03	1.05	0.10	0.823	0.420	0.263
Ash	1.49	1.42	1.53	1.47	1.53	1.41	0.07	0.331	0.080	0.690

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA).

Table 4: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on antioxidant indices of hepatopancreas and hemolymph of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
Hepatopancreas										
T-AOC (U/mg protein)	4.04^{a}	7.19^{bc}	4.04^{a}	5.52^{ab}	10.45^{d}	8.13^{c}	1.02	<0.001	<0.001	<0.001
GSH-Px (U/mg protein)	9.98^{b}	11.14^{bc}	9.93^{b}	8.74^{a}	11.83^{c}	11.29^{c}	0.82	0.417	<0.001	0.008
MDA (nmol/mg protein)	1.85^{a}	1.36^{a}	1.60^{a}	1.54^{a}	1.48^{a}	2.35^{b}	0.33	0.115	0.003	0.003
SOD (U/mg protein)	10.34	10.17	8.96	9.62	10.89	11.64	1.64	0.157	0.769	0.098
CAT (U/mg protein)	2.72^{a}	3.83^{ab}	2.93^{ab}	4.39^{c}	6.22^{d}	4.51^{c}	0.96	<0.001	0.003	0.525
Hemolymph										
T-AOC (U/mL)	8.23^{a}	8.81^{a}	8.76^{a}	9.30^{bc}	10.61^{cd}	11.07^{d}	0.77	<0.001	0.019	0.301
GSH-Px (U/mL)	49.83^{ab}	56.13^{c}	53.48^{bc}	45.92^{a}	53.62^{bc}	48.00^{a}	2.56	0.001	<0.001	0.538
MDA (nmol/mL)	6.11^{bc}	3.49^{a}	5.24^{b}	6.43^{c}	3.02^{a}	5.11^{b}	0.77	0.469	<0.001	0.686
SOD (U/mL)	42.71	37.12	37.40	41.07	41.36	38.66	4.46	0.453	0.172	0.384
CAT (U/mL)	8.92	8.96	8.02	8.36	10.30	10.39	0.60	0.178	0.551	0.287

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). T-AOC: total antioxidant capacity; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase.

Table 5: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on nonspecific immune indices and physiological status of hepatopancreas and hemolymph of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
Hepatopancreas										
AKP (U/mg protein)	57.28	61.47	49.32	46.22	57.26	54.82	10.95	0.410	0.216	0.238
ACP (U/mg protein)	5.48^{ab}	6.54^{b}	4.83^{a}	6.27^{ab}	5.63^{ab}	6.28^{ab}	0.85	0.172	0.391	0.015
GPT (U/mg protein)	5.23^{cd}	4.38^{bc}	5.02^{cd}	5.86^{d}	3.65^{ab}	3.02^{a}	0.60	0.016	<0.001	0.002
GOT (U/mg protein)	4.39^{b}	2.53^{a}	4.10^{b}	4.07^{b}	2.10^{a}	1.66^{a}	0.70	<0.001	0.004	0.285
Hemolymph										
AKP (U/100 mL)	1.51^{a}	1.60^{ab}	1.69^{ab}	2.52^{c}	2.08^{bc}	1.35^{a}	0.28	0.002	0.006	<0.001
ACP (U/100 mL)	2.23^{a}	2.19^{a}	2.52^{ab}	4.07^{c}	3.01^{b}	3.09^{b}	0.44	<0.001	0.048	0.016
GPT (U/mL)	8.87^{c}	5.99^{b}	7.32^{bc}	3.10^{a}	1.82^{a}	1.77^{a}	1.79	<0.001	<0.001	0.002
GOT (U/mL)	19.54^{b}	18.99^{b}	19.98^{b}	10.00^{a}	13.71^{a}	12.14^{a}	0.84	<0.001	0.414	0.259

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). AKP: alkaline phosphatase; ACP: acid phosphatase; GPT: glutamic pyruvic transaminase; GOT: glutamic oxalacetic transaminase.

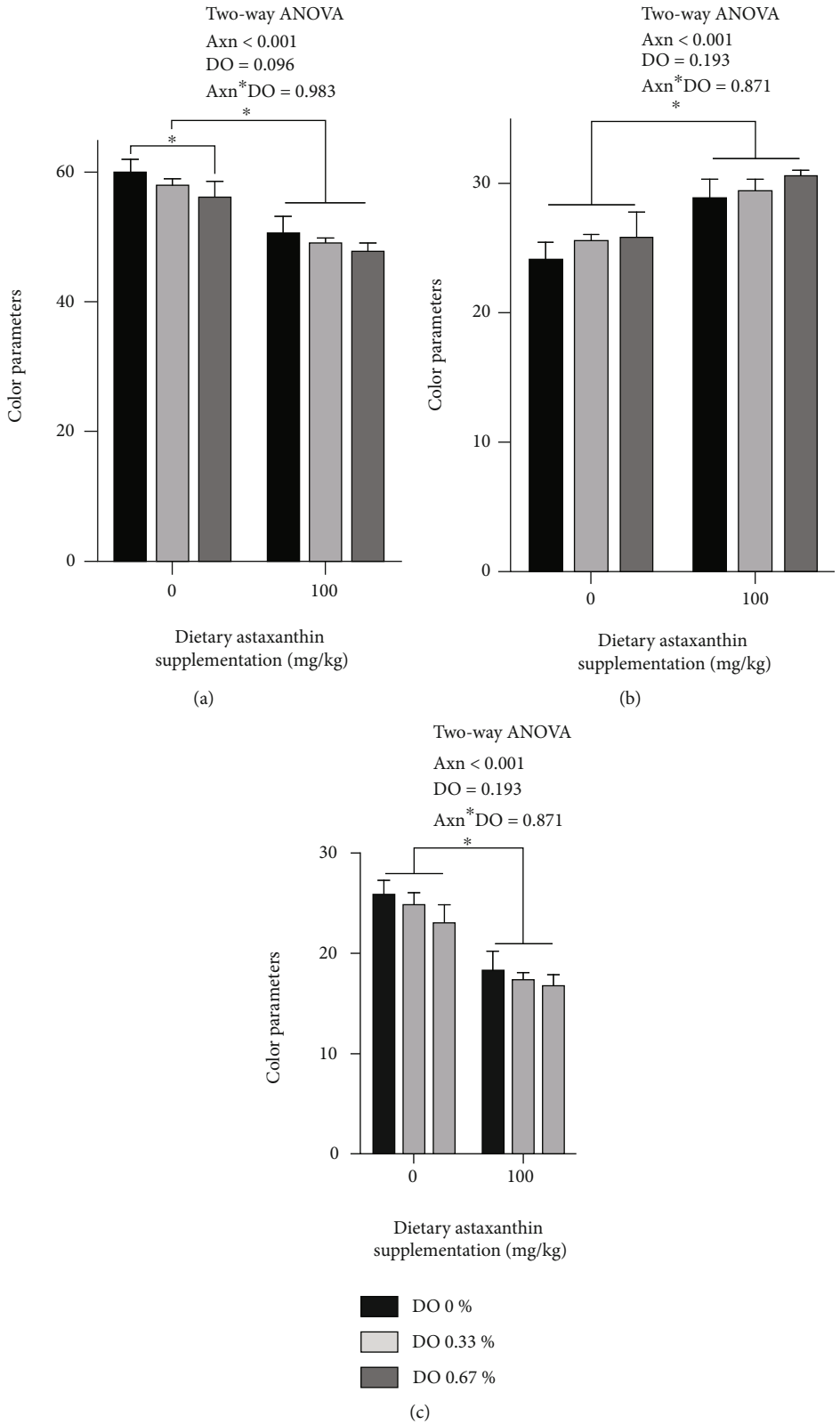

Figure 1: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on ovary coloration of adult female *E. sinensis* ($n = 3$). The data are expressed as the means ± standard deviation (SD). Bars with "*" mean significant difference ($P < 0.05$): (a) lightness; (b) redness; (c) yellowness.

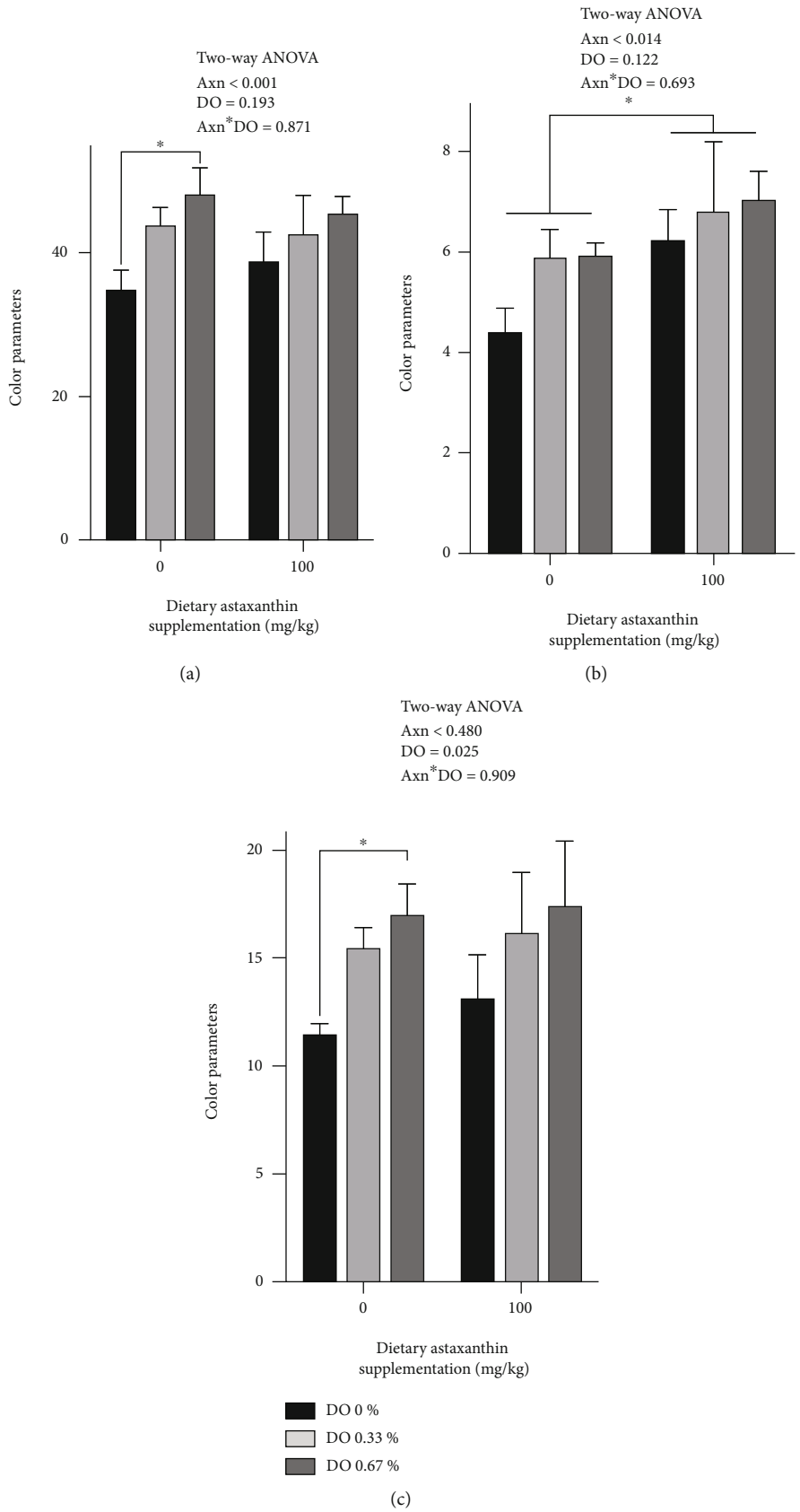

Figure 2: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on hepatopancreas coloration of adult female *E. sinensis* ($n = 3$). The data are expressed as the means ± standard deviation (SD). Bars with "*" mean significant difference ($P < 0.05$): (a) lightness; (b) redness; (c) yellowness.

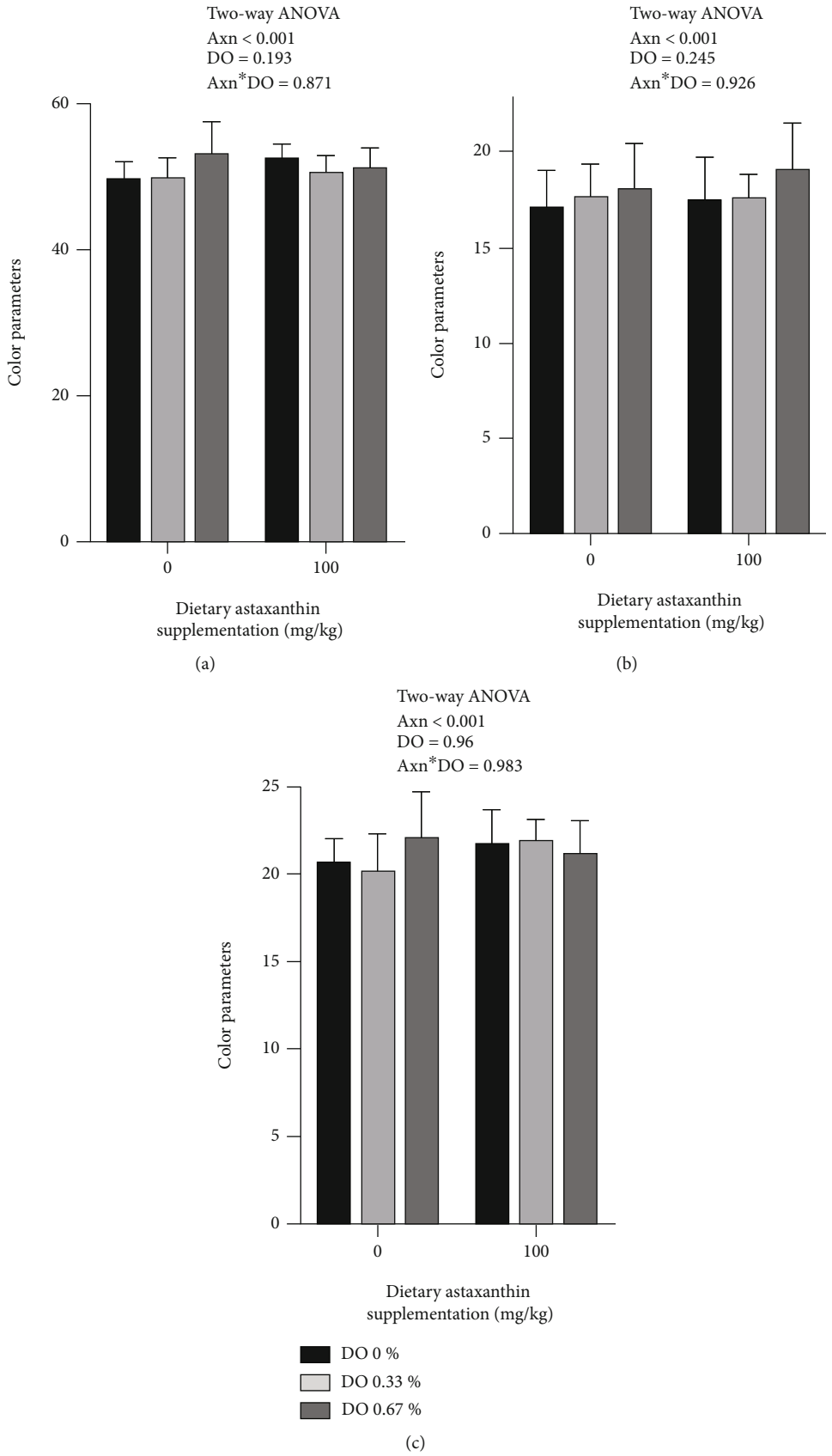

Figure 3: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on carapace coloration of adult female *E. sinensis* ($n = 3$). The data are expressed as the means ± standard deviation (SD): (a) lightness; (b) redness; (c) yellowness.

Table 6: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on carotenoid contents in ovaries, hepatopancreas, and carapace of male and female *E. sinensis* ($n = 3$).

Items (mg/kg)	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
Ovaries										
Total carotenoids	257.95^{a}	338.56^{a}	338.84^{a}	494.04^{b}	538.14^{b}	558.25^{b}	45.55	<0.001	0.059	0.824
Astaxanthin	43.77^{a}	47.07^{a}	53.96^{a}	266.29^{b}	300.89^{b}	379.44^{c}	23.09	<0.001	0.059	0.824
Lutein	3.62	4.21	3.40	4.07	3.47	4.55	0.70	0.429	0.941	0.130
Zeaxanthin	11.59^{a}	11.91^{a}	11.11^{a}	14.37^{b}	14.64^{b}	15.40^{b}	1.06	<0.001	0.878	0.417
β-Carotene	49.21	52.21	41.02	44.95	44.16	40.92	9.97	0.392	0.422	0.788
Hepatopancreas										
Total carotenoids	57.06^{a}	73.84^{a}	84.37^{a}	188.90^{b}	211.11^{b}	228.20^{b}	18.48	<0.001	0.033	0.865
Astaxanthin	2.26^{a}	2.36^{a}	2.50^{a}	13.11^{b}	16.20^{c}	18.28^{c}	0.97	<0.001	0.002	0.004
β-Carotene	26.73^{a}	34.39^{a}	39.90^{a}	82.86^{b}	91.72^{b}	106.04^{b}	11.34	<0.001	0.101	0.782
Carapace										
Total carotenoids	20.39	25.97	34.78	25.11	30.30	33.34	7.65	0.495	0.074	0.743
Astaxanthin	10.09	12.86	13.17	15.03	17.67	20.68	3.95	0.045	0.036	0.866
Lutein	1.70	1.81	1.70	1.94	2.07	2.29	0.37	0.067	0.724	0.686
Zeaxanthin	1.54	1.44	1.27	2.81	2.74	2.41	1.21	0.073	0.902	0.994
β-Carotene	1.82	1.55	0.73	1.28	0.46	1.85	0.44	0.551	0.290	0.016

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). Carotenoids less than 0.3 mg/kg were not listed in the table.

Table 7: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on lipid class composition (mg/g wet weight) of ovaries, hepatopancreas, and carapace of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
Ovaries										
Triacylglycerol	69.71	74.09	74.78	78.18	73.59	68.22	4.73	0.880	0.759	0.165
Free fatty acids	1.78	2.93	1.38	1.76	0.49	1.41	1.19	0.267	0.896	0.289
Cholesterol	1.89	3.35	3.22	2.00	1.16	1.64	1.24	0.160	0.888	0.509
Phospholipids	81.48	81.03	84.46	83.79	80.14	72.75	4.92	0.266	0.551	0.168
Hepatopancreas										
Triacylglycerol	55.01^{a}	115.13^{c}	116.61^{c}	98.00^{b}	104.92^{b}	103.45^{b}	2.57	0.001	<0.001	<0.001
Free fatty acids	0.43^{a}	0.70^{ab}	1.09^{ab}	1.44^{b}	0.27^{a}	0.25^{a}	0.35	0.700	0.281	0.012
Cholesterol	0.23	0.72	0.57	0.55	0.69	0.36	0.31	0.886	0.311	0.456
Phospholipids	8.71	9.95	10.14	12.98	11.23	11.32	2.66	0.224	0.993	0.721
Muscle										
Triacylglycerol	1.19	1.26	1.31	1.29	1.26	1.35	0.07	0.437	0.410	0.805
Free fatty acids	0.98	1.02	1.02	1.01	1.00	1.04	0.07	0.898	0.788	0.902
Cholesterol	0.54	0.62	0.56	0.52	0.53	0.53	0.06	0.203	0.601	0.644
Phospholipids	7.61	7.33	7.88	7.89	7.53	7.57	0.10	0.574	0.032	0.049

All data are expressed as means with pooled standard deviation (SD). Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA).

also significantly higher in the three Axn-supplemented diets (Diets 4-6), in which feeding 0.33% and 0.67% DHA oil (Diets 5-6) produced a further significant improvement in hepatosomatic Axn concentration compared to the treatment supplemented with Axn only (Diet 4). Carotenoid concentration in the carapace did not differ significantly among all treatments ($P > 0.05$).

3.4. Lipid Class and Fatty Acid Composition. The main lipid class compositions based on the wet weight of the tissues are shown in Table 7. TG is the major lipid class in the hepatopancreas (85-90%), and its contents were significantly higher in Diets 2-6 treatments, with a significant increase from dietary DHA supplementation ($P < 0.05$). Dietary variables had no markable effect on lipid class composition of the ovaries and muscle. For lipid class compositions based on the total lipid class of tissues, a significant difference was observed only in the hepatosomatic TG composition, which was significantly higher in DHA-supplemented treatments, regardless of the presence of dietary Axn supplementation (Supplementary Table 2).

Table 8 shows the fatty acid composition and percentage in the ovaries. No significant difference was detected in the percentages of saturated fatty acids (SFAs) among all treatments. Within mono-unsaturated fatty acids (MUFAs), the percentages of C16:1n7, C18:1n7, and Σ MUFAs showed a decreasing trend with DHA oil supplementation from 0% to 0.67%, while supplementation with 100 mg/kg Axn significantly decreased the percentages of C18:1n7 and Σ MUFAs in the ovaries ($P < 0.05$). Fewer significant differences occurred in polyunsaturated fatty acids (PUFAs). The percentage of C20:4n6 showed a "low-high-low" pattern with the increase in dietary DHA supplementation, and C22:6n3 percentage was significantly higher in the crabs fed diets supplemented with 0.33% and 0.67% DHA oil ($P < 0.05$).

Crabs fed diets supplemented with 100 mg/kg Axn (Diet 4-6) had comparatively lower percentages of C18:0 and Σ SFAs in their hepatopancreas (Table 9). For MUFAs, dietary Axn and DHA noticeably decreased C16:1n7 percentage in the hepatopancreas, and the highest percentage was observed in Diet 5 treatment. In contrast, Axn and DHA supplementation increased C18:1n7 percentage, and the lowest percentage was found in Diet 6 treatment. Most differences were observed in the PUFAs. Dietary Axn or DHA significantly decreased C18:2n6 percentages, and Diet 5 treatment yielded a significantly higher C20:2n6 percentage than other five treatments ($P < 0.05$). Moreover, C22:6n3 and Σ PUFAs percentages showed an increasing trend with DHA supplementation, regardless of the presence of dietary Axn supplementation. As for the indices describing combinations of PUFAs, Σ n-3PUFAs and Σ LC-PUFAs showed an increasing trend with DHA supplementation, and dietary supplementation with Axn and DHA significantly increased Σ n-6PUFA and DHA/EPA levels in the hepatopancreas ($P < 0.05$).

Table 10 shows the fatty acid composition and percentage within the muscle. Within SFAs, the percentages of C16:0, C18:0, and Σ SFA were all significantly higher in Diet 1 treatment. The significant difference within MUFAs was only observed in C18:1n7 which significantly decreased with the increase in dietary DHA supplementation. For differences within PUFAs, Diet 6 had a significantly higher C20:4n6 percentage than Diet 3, but significantly lower C22:5n3 percentage than Diet 4 ($P < 0.05$). Dietary DHA supplementation contributed to higher percentages of C22:6n3 and Σ PUFAs, but no significant difference was detected ($P > 0.05$).

4. Discussion

GSI is a reliable indicator for evaluating the ovarian development of female *E. sinensis* [50, 51]. Supplementation with 0.33% DHA oil significantly increased the GSI of female crabs in this study, and the demand for DHA during ovarian development may stem from the need for maternal DHA during vitellogenesis and yolk formulation [34, 52]. Dietary Axn had no apparent effects on GSI among treatments, agreed with previous studies that have reported in *E. sinensis* [7, 35] and *P. trituberculatus* [53]. Crabs used for feeding trial in present study had completed their final molt (puberty molt); therefore, they only had approximately 5.5% of GSI after the 70-day indoor culture. Hepatopancreas is an important organ for both absorption and storage of consumed lipids [54]. Although HSI exhibited no significant difference in present study, Axn and DHA both significantly increased lipid and ash contents in the hepatopancreas. The promotion of nutrient accumulation by dietary Axn agreed with previous research on swimming crab *P. trituberculatus* that hepatopancreatic lipid content was significantly elevated by 40-80 mg/kg Axn [42]. A possible explanation for this increase in hepatopancreatic lipid content could be that the oxidative stress and energy expenditure were reduced by deposited Axn [7].

Axn is characterised by its antioxidant properties arising from its unique molecular structure that quenches singlet oxygen and free radicals [55, 56]. In this study, supplementation with 100 mg/kg Axn significantly increased T-AOC, AKP, and ACP levels in the tissues, demonstrating a positive effect on antioxidant status and immune response of fed crabs. GPT and GOT are important amino acid transaminases that play central roles in protein metabolism and immunity [57]. The activity of GPT and GOT is sensitive indicators of the physiological status of hepatocytes, and the high levels of GPT and GOT in the serum suggest the possible pathology (i.e., inflammation, degeneration, and necrosis) in the liver or hepatopancreas [58]. The supplementation of 100 mg/kg Axn markedly decreased GOT and GPT levels in the hemolymph of present study, indicating that Axn supplementation has a protective effect against hepatopancreatic damage and cell death in *E. sinensis*, which could be explained by the antioxidant capability and immunity preservation characteristics of dietary Axn [45].

There are inconsistent reports on the effects of dietary n-3 PUFAs on antioxidant capability of different aquatic animals. Some experiments showed the protection of n-3 PUFAs against oxidative stress [59, 60], while others reported lipid peroxidation induced by dietary n-3 PUFAs supplementation [61, 62]. In this study, regardless of the

Table 8: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on fatty acid composition (% of total fatty acid) in the ovaries of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
C14:0	0.57	0.56	0.60	0.57	0.55	0.56	0.04	0.706	0.881	0.929
C16:0	10.35	9.17	9.65	9.68	9.32	9.54	0.40	0.587	0.314	0.691
C18:0	2.84	2.65	2.78	2.87	2.53	2.61	0.12	0.466	0.238	0.748
Σ SFA	14.51	13.35	13.94	13.80	13.45	13.45	0.46	0.489	0.516	0.81
C16:1n7	3.08^{c}	2.47^{ab}	2.55^{ab}	2.82^{bc}	2.64^{bc}	2.10^{a}	0.12	0.163	0.005	0.140
C18:1n9	18.31	15.91	16.95	17.00	16.43	16.08	0.59	0.400	0.196	0.509
C18:1n7	2.97^{b}	2.87^{ab}	2.72^{ab}	2.79^{ab}	2.46^{ab}	2.18^{a}	0.16	0.047	0.142	0.677
C20:1n9	1.24^{b}	1.18^{b}	1.25^{b}	1.30^{b}	0.62^{a}	0.74^{a}	0.08	0.004	0.021	0.033
Σ MUFA	25.99^{b}	22.88^{ab}	23.85^{ab}	24.36^{ab}	22.54^{a}	21.45^{a}	0.76	0.090	0.041	0.561
C18:2n6	12.23	11.40	12.13	12.48	11.64	11.54	0.38	0.935	0.253	0.573
C18:3n3	0.53	0.47	0.49	0.51	0.56	0.39	0.07	0.890	0.630	0.631
C20:2n6	0.93	0.83	0.86	0.62	0.96	0.94	0.12	0.847	0.861	0.658
C20:4n6	0.89^{a}	1.21^{bc}	1.02^{b}	0.96^{ab}	1.30^{c}	0.88^{a}	0.07	0.926	0.009	0.388
C20:5n3	4.17	4.50	4.12	4.09	4.66	4.19	0.38	0.889	0.500	0.961
C22:6n3	2.21^{a}	4.10^{c}	3.61^{bc}	2.71^{ab}	3.76^{bc}	4.07^{c}	0.26	0.521	0.007	0.515
Σ PUFA	21.10	22.66	22.49	21.78	23.05	22.23	0.87	0.755	0.422	0.886
Σ n-3PUFA	7.53	10.00	9.05	8.35	10.13	9.46	0.59	0.512	0.084	0.92
Σ n-6PUFA	14.16	13.58	14.16	14.43	13.96	13.56	0.44	0.969	0.594	0.588
Σ LC-PUFA	8.27	10.72	9.80	8.47	10.83	10.14	0.58	0.735	0.033	0.987
DHA/EPA	0.53^{a}	0.91^{c}	0.86^{c}	0.66^{ab}	0.82^{bc}	0.98^{c}	0.03	0.255	0.008	0.084

All data are expressed as means with pooled standard deviation (SD). Fatty acids less than 0.3% were not listed in the table. Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ PUFA: total polyunsaturated fatty acids; Σ n-3PUFA: total n-3 polyunsaturated fatty acids; Σ n-6PUFA: total n-6 polyunsaturated fatty acids; Σ LC-PUFA: total long chain polyunsaturated fatty acids.

Table 9: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on fatty acid composition (% of total fatty acid) in the hepatopancreas of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P*		
								Axn	DO	Axn × DO
C14:0	0.95	1.11	1.19	0.95	1.11	1.10	0.05	0.565	0.050	0.761
C16:0	12.28	12.23	12.84	11.90	12.39	11.73	0.44	0.199	0.841	0.297
C18:0	3.10^{b}	2.42^{a}	2.72^{ab}	2.44^{a}	2.35^{a}	2.31^{a}	0.14	0.009	0.075	0.188
C20:0	0.61	0.41	0.47	0.33	0.37	0.35	0.08	0.064	0.651	0.441
Σ SFA	17.99^{bc}	17.05^{bc}	18.16^{c}	16.46^{ab}	17.06^{ab}	16.37^{a}	0.49	0.017	0.894	0.172
C16:1n7	2.52^{a}	3.12^{bc}	2.82^{ab}	2.68^{ab}	3.24^{c}	2.99^{bc}	0.14	0.247	0.013	0.978
C18:1n9	23.58	24.69	24.69	22.81	24.83	24.10	0.78	0.504	0.135	0.811
C18:1n7	2.81^{b}	2.61^{ab}	2.66^{ab}	2.68^{ab}	2.49^{ab}	2.36^{a}	0.11	0.092	0.164	0.696
C20:1n9	2.57	2.31	2.35	2.14	2.24	2.40	0.13	0.170	0.704	0.186
C22:1n9	1.37	1.47	1.53	1.40	1.56	1.63	0.15	0.570	0.462	0.964
Σ MUFA	33.31	34.65	34.46	32.13	34.78	33.90	0.99	0.504	0.154	0.802
C18:2n6	8.59^{a}	10.55^{b}	10.07^{b}	12.00^{b}	10.43^{b}	10.79^{b}	0.73	0.014	0.042	0.128
C18:3n3	0.59	0.79	0.80	0.87	0.87	0.84	0.08	0.096	0.353	0.509
C20:2n6	1.52^{a}	1.46^{a}	1.29^{a}	1.49^{a}	1.57^{a}	2.11^{b}	0.10	0.005	0.007	0.064
C20:4n6	0.90^{b}	0.67^{ab}	0.78^{ab}	0.56^{a}	0.83^{ab}	0.83^{ab}	0.09	0.553	0.058	0.627
C20:5n3	1.34	1.12	1.31	0.75	1.39	1.39	0.20	0.633	0.085	0.464
C22:5n3	0.40^{ab}	0.42^{ab}	0.25^{a}	0.33^{ab}	0.45^{ab}	0.59^{b}	0.09	0.214	0.258	0.215
C22:6n3	2.17^{a}	3.27^{ab}	3.21^{ab}	2.25^{a}	3.53^{ab}	4.01^{b}	0.39	0.223	0.068	0.024
Σ PUFA	15.62^{a}	18.46^{ab}	17.85^{ab}	18.38^{ab}	19.25^{ab}	20.73^{b}	1.29	0.025	0.426	0.088
Σ n-3PUFA	4.61^{ab}	5.77^{b}	5.70^{b}	4.33^{a}	6.42^{bc}	7.01^{c}	0.73	0.301	0.171	0.061
Σ n-6PUFA	11.01^{a}	12.69^{b}	12.14^{b}	14.05^{b}	12.83^{b}	13.72^{b}	0.71	0.010	0.156	0.114
Σ LC-PUFA	6.44^{ab}	7.11^{ab}	6.97^{ab}	5.51^{a}	7.95^{b}	9.10^{b}	0.70	0.228	0.114	0.039
DHA/EPA	1.65^{a}	2.91^{b}	2.57^{b}	3.02^{b}	2.60^{b}	2.97^{b}	0.14	0.035	0.014	0.245

All data are expressed as means with pooled standard deviation (SD). Fatty acids less than 0.3% were not listed in the table. Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). Σ SFA: total saturated fatty acids; Σ MUFA: total mono unsaturated fatty acids; Σ PUFA: total polyunsaturated fatty acids; Σ n-3PUFA: total n-3 polyunsaturated fatty acids; Σ n-6PUFA: total n-6 polyunsaturated fatty acids; Σ LC-PUFA: total long chain polyunsaturated fatty acids.

Table 10: Effects of dietary astaxanthin (Axn) and DHA oil (DO) on fatty acid composition (% of total fatty acid) in the muscle of adult female *E. sinensis* ($n = 3$).

Items	Diet-1 Axn 0 + DO 0	Diet-2 Axn 0 + DO 0.33	Diet-3 Axn 0 + DO 0.67	Diet-4 Axn 100 + DO 0	Diet-5 Axn 100 + DO 0.33	Diet-6 Axn 100 + DO 0.67	SD	*P* Axn	*P* DO	*P* Axn × DO
C16:0	6.61^{b}	6.64^{b}	5.96^{a}	6.01^{a}	5.95^{a}	5.92^{a}	0.14	0.009	0.091	0.176
C18:0	4.26^{b}	4.03^{ab}	3.74^{a}	4.00^{ab}	3.96^{ab}	3.93^{ab}	0.14	0.676	0.097	0.219
C20:0	0.59	0.39	0.69	0.70	0.53	0.60	0.11	0.623	0.326	0.667
C22:0	0.36	0.31	0.30	0.32	0.31	0.32	0.02	0.609	0.422	0.444
Σ SFA	12.46^{b}	11.99^{ab}	11.18^{a}	11.59^{ab}	11.30^{a}	11.29^{a}	0.21	0.086	0.081	0.290
C16:1n7	0.74	0.86	0.79	0.78	0.83	0.77	0.03	0.826	0.156	0.627
C18:1n7	1.56^{c}	1.49^{bc}	1.36^{ab}	1.58^{c}	1.37^{ab}	1.29^{a}	0.03	0.128	0.001	0.323
C18:1n9	10.23	10.24	9.73	9.29	9.81	9.56	0.33	0.122	0.597	0.604
C20:1n9	0.62	0.59	0.74	0.58	0.69	0.71	0.04	0.791	0.055	0.294
Σ MUFA	13.29	13.32	12.73	12.36	12.82	12.45	0.36	0.129	0.552	0.757
C18:2n6	6.22	6.74	6.76	5.96	7.04	6.20	0.37	0.633	0.228	0.617
C18:3n3	0.54	0.59	0.51	0.61	0.62	0.57	0.07	0.417	0.760	0.963
C20:2n6	2.01	1.74	2.21	2.23	1.97	2.15	0.15	0.335	0.155	0.636
C20:4n6	2.23^{ab}	2.06^{ab}	1.96^{a}	2.23^{ab}	2.07^{ab}	2.49^{b}	0.11	0.121	0.402	0.120
C20:5n3	8.71	8.94	8.73	8.48	9.10	9.33	0.56	0.762	0.789	0.842
C22:5n3	0.35^{ab}	0.28^{ab}	0.27^{ab}	0.39^{b}	0.28^{ab}	0.26^{a}	0.03	0.796	0.028	0.778
C22:6n3	8.04	8.87	8.34	7.56	8.80	8.69	0.50	0.879	0.205	0.761
Σ PUFA	28.32	29.44	29.02	27.72	30.12	29.96	1.20	0.802	0.520	0.876
Σ n-3PUFA	17.86	18.89	18.10	17.29	19.04	19.12	0.97	0.849	0.522	0.812
Σ n-6PUFA	10.46	10.54	10.93	10.43	11.08	10.84	0.39	0.703	0.588	0.743
Σ LC-PUFA	21.56	22.11	21.75	21.14	22.46	23.19	1.00	0.647	0.613	0.741
DHA/EPA	0.93	0.99	0.96	0.90	0.97	0.93	0.03	0.313	0.196	0.989

All data are expressed as means with pooled standard deviation (SD). Fatty acids less than 0.3% were not listed in the table. Values in the same row without a common letter are significantly different ($P < 0.05$, one-way ANOVA). Σ SFA: total saturated fatty acids; Σ MUFA: total monounsaturated fatty acids; Σ PUFA: total polyunsaturated fatty acids; Σ n-3PUFA: total n-3 polyunsaturated fatty acids; Σ n-6PUFA: total n-6 polyunsaturated fatty acids; Σ LC-PUFA: total long chain polyunsaturated fatty acids.

presence of dietary Axn supplementation, the provision of 0.33% DHA oil produced a significant decrease in MDA content in the hemolymph, whereas a further increase in dietary DHA oil from 0.33% to 0.67% led to a significant increase in hemolymph MDA content. These results suggest that the antioxidant capacity of *E. sinensis* was elevated by 0.33% DHA oil, but excessive dietary DHA oil (0.67%) supplementation would increase hepatic lipid peroxidation [55, 63]. Axn is known to powerfully cleave singlet oxygen and possesses the physiological property of protecting many tissues from lipid peroxidation, especially peroxidation of PUFAs in the diet [64]. In the Diets 4-5 treatments of the present study, MDA concentration in the hepatopancreas and hemolymph both showed an apparent "high-low-high" pattern with DHA oil supplementation from 0% to 0.67%, indicating that 100 mg/kg of Axn was unable to prevent the lipid peroxidation induced by 0.67% DHA oil. A possible reason for this could be that supplementation with 0.67% DHA oil is excessive for any apparent antioxidant capacity exerted by the deposited Axn [65]. In comparison, 100 mg/kg Axn in combination with 0.33% DHA oil (Diet 5) may have more antioxidant effects, but it is difficult to estimate how much of this promising evidence is due to the direct antioxidant property of Axn or DHA, and how much is due to their synergistic promotion on *E. sinensis* health [66, 67].

Crustaceans have the capacity to store a great quantity of carotenoids or convert these carotenoids into Axn before depositing in the tissues [68, 69]. In crustaceans, the content of deposited Axn correlates well with tissue redness, with many studies reporting redness values as an intuitive predictor of tissue Axn concentrations [10, 70]. As expected, Axn concentration in the ovaries and hepatopancreas increased synchronously with tissue redness (a∗) in the present study. Dietary supplementation of 30-120 mg/kg Axn was reported to raise redness and Axn concentration in the shell of juvenile *E. sinensis* [6] and adult *E. sinensis* [7, 35], but no such relationship was found in the present study. We hypothesise that these differences in *E. sinensis* carapace pigmentation are directly related to the different days of postpuberty moulting of the experimental crabs [14, 71]. For defence and locomotion, during the postmolt stage of crustacean animals, the exoskeleton of the new carapace hardens quickly via sclerotization and mineralisation within 24 h. Furthermore, the calcification and carotenoid recovery of the new carapace is just about finished within 96 h after molting [72, 73]. The experimental crabs completed puberty molting approximately 15 days before the beginning of the growth trial in present study; thus, dietary Axn supplementation had less of a promoting effect on carapace colouration.

The retention efficiency of dietary Axn depends on many factors besides source and inclusion levels, such as the types and levels of dietary lipids, which may influence Axn bioavailability and tissue absorption [74]. In this study, DHA supplementation significantly enhanced Axn deposition in the ovaries and hepatopancreas of *E. sinensis*. The reason for the positive effects of dietary DHA on Axn deposition could be explained by the following facts: (1) Synthetic Axn is all in its free form and thus is vulnerable to oxidation, but the esterification of free Axn with LC-PUFA (monoesters and diesters) may improve molecular stability and facilitate higher solubility rates in crustacean tissues [75–77]. Moreover, dietary supplementation of DHA oil facilitates the esterification of free Axn in animal tissue, which may lead to increased Axn deposition in the tissues of *E. sinensis*. (2) The hydroxyl groups present in the Axn molecule provide polar characteristics that have an affinity for the polar heads of DHA [78]. (3) DHA oil may act as an emulsifier making the digestion and absorption of Axn easier [79].

Decapod hepatopancreas and gonad are the major organs for fatty acid metabolism; therefore, they are highly influenced by dietary fatty acid profiles [80–82]. In present research, the DHA percentage and DHA/EPA ratio in the gonads and hepatopancreas significantly increased with dietary DHA supplementation. Studies have provided evidence that dietary Axn supplementation increased DHA and EPA percentages in *P. trituberculatus* [83] and *P. monodon* [84] and speculated that Axn decreased lipid peroxidation and oxidation of these fatty acids. However, when compared across treatments in this study, DHA percentage was not significantly affected by dietary Axn supplementation. These differences in DHA accumulation could be a result of specific developmental stages or different potential methodologies [14, 71]. Finally, our results showed that dietary Axn and DHA had little influence on the lipid class and fatty acid composition within the muscle, suggesting that the muscles of adult *E. sinensis* individuals are generally more stable than the hepatopancreas and ovaries during the ovarian maturation stage [33, 36]).

5. Conclusion

The gonad development of *E. sinensis* in present study demonstrated that dietary supplementation with both DHA oil and Axn significantly improved the GSI and hepatosomatic lipid content in adult females of *E. sinensis*. Supplementation with both 0.33% DHA oil and 100 mg/kg Axn also dramatically promoted the antioxidative capacity, nonspecific immunity, and physiological status of adult females of *E. sinensis*, but supplementation with 0.67% DHA oil induced negative effects on the health status of the crabs. The supplementation of 100 mg/kg Axn in the diets dramatically increased the redness (a∗) and Axn concentration in both the ovaries and hepatopancreas, and supplementation with 0.33% or 0.67% DHA oil further increased Axn concentration in the tissues. Overall, present study provides clear evidence that interactions between Axn and DHA indeed occur, and the optimal combination of dietary Axn and DHA oil was around 100 mg/kg and 0.33% for female *E. sinensis* during gonad development stage.

Data Availability

The data could be made available on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiaodong Jiang and Kewu Pan contributed equally to this work.

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Supplementary Materials

Supplementary Table 1: proximate composition and major carotenoid concentration of the experimental diets. Supplementary Table 2: effects of dietary astaxanthin (Axn) and DHA oil (DO) on lipid class composition (% total lipid class) of ovaries, hepatopancreas, and carapace of adult female *E. sinensis*. *(Supplementary Materials)*

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