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Research Article

The Effect of Dietary Supplementation with *Haematococcus pluvialis* for Enhanced Pigmentation in *Amphiprion ocellaris*

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Among the minority seawater species in the ornamental fish market, clownfish (*Amphiprion ocellaris*) have a high market value due to their vibrant appearance. Astaxanthin, a promising coloring agent, is applied in diets as a food additive to enhance the physical appearance of clownfish. However, as the retail price of astaxanthin has been inflating, the industry is eager to find more affordable astaxanthin products. The present study evaluated the nutrients and pigmentation performance of the algae-extracted astaxanthin diets of clownfish, followed by a comparative feeding trial evaluating the graded levels of either *Haematococcus pluvialis* powder or extracted astaxanthin supplements in the experimental diets. The study included three stages: assessing the utilization of *H. pluvialis* powder as an astaxanthin source, investigating the optimal practical extraction method, and revisiting the effects of crude extracted astaxanthin on clownfish based on the identified preparation processes. In the first stage, *H. pluvialis* powder was applied as the feed additive for a feeding trial, resulting in a low-utilization rate of astaxanthin in clownfish. This indicated that a suitable extraction method was essential for clownfish. Two chemical-based methods for astaxanthin extraction were investigated to obtain the optimal extraction yields; results showed that the HCl–acetone method had an astaxanthin yield of 21.99 ± 0.52 mg/g cell, which was significantly higher than the yield from the acetic acid-DMSO method. Finally, the effects of HCl–acetone-extracted astaxanthin was compared with the effects of synthesized astaxanthin. The pigmentation performance study was performed using a digital image acquisition and processing technique. A significant increase in the red color signal was observed at week six after fed with 400 and 200 mg/kg of the extracted astaxanthin, compared to the control 0 mg/kg. In conclusion, *H. pluvialis*-extracted astaxanthin following the HCl–acetone method can significantly improve the pigmentation performance of *A. ocellaris* after 42 days of oral administration.

1. Introduction

The market demand for ornamental fish, which has a worldwide industry value of over 15-billion USD, is continuously increasing [1]. According to a business research report, clownfish has a market size 112-million USD in 2022 and estimated to reach 176-million USD in 2028 [2]. To satisfy this demand, artificial cultural techniques have been developed to achieve a stable supply and eliminate the environmental impacts of wild collection [3]. However, the production of ornamental fish is facing the issue of decoloration in the offspring due to the simplicity of the nutrients supplied, cultural environment, and genetic attenuation, thereby affecting the market value [4]. Compared with the artificial breeds, wild fish accumulate pigments by digesting microalgae and zooplankton from their habitats, which are then reflected on their appearance [5, 6]. To amend the decoloration problems, diets supplied with the pigmentation enhancers are regarded as the most practical way to improve the physical appearance of an ornamental fish [7–9].

Astaxanthin, initially utilized in salmon to enhance their muscle pigmentation performance, has been reported in recent decades to be a promising bioactive compound with

an outstanding anti-oxidation and bacterial resistance and further applied for nutraceutical and cosmetic purposes [7, 10, 11]. However, an increase in the retail price of artificially synthesized astaxanthin has increased the production costs for aquaculture farmers. The industry is thus eager to find an affordable alternative resource. Instead of being artificially synthesized, astaxanthin can be naturally produced by microalgae, fungi, bacteria, or crustaceans, which create over 300 tons of astaxanthin a year [12, 13]. In the past, the utilization of naturally derived astaxanthin has been limited by poor extraction efficiency [14].

Microalgae *Haematococcus pluvialis* (Chlorophyta) has been discovered to have a high-astaxanthin production efficiency, as it can accumulate astaxanthin up to 4% of its dry weight when its algae cells experience environmental stress [15, 16]. However, as a part of the microalgae's adversity mechanism, astaxanthin accumulation comes with a thick amorphous layer that requires additional processing before collection [17, 18]. The current strategies for the breakdown of *H. pluvialis* red cyst cells include physical and chemical methods, but the efficiency still needs improvement [19, 20].

The complete artificial breeding techniques of clownfish (*Amphiprion ocellaris*) have been developed in the commercial farming industry. Although clownfish production has developed, fish from hatcheries still have a decoloration issue compared to the wild-caught fish. Like most aquatic animals, clownfish have no carotenoid biosynthesis ability. The pigmentation relies on diet or the accumulation of other exogenous resources [21]. Several studies have reported that the oral administration of dietary carotenoids, including astaxanthin, beta-carotene, and canthaxanthin, can significantly increase the pigmentation performance of clownfish [22, 23]. By using supplements of artificially synthesized astaxanthin, the skin color of clownfish can be improved [24].

The present study includes the following three steps: first, evaluating the utilization of lyophilized *H. pluvialis* by oral administration for pigmentation enhancement; second, investigating the optimal extraction methods of astaxanthin from the *H. pluvialis* powder; finally, comparing the effects of the extracted natural astaxanthin with those of the synthesized astaxanthin. By developing the best preparation protocol, from algae enrichment to astaxanthin extraction, the related products can become more practical and cost-effective.

2. Materials and Methods

2.1. Fish. Experimental clownfish larvae were obtained from the National Taiwan Ocean University's (NTOU) aquaculture center, accumulated in an indoor recirculating culture system (RAS), and fed a commercial diet twice a day before the study. Water quality was monitored daily with temperature, salinity (WQC-30, TOA-DKK, Tokyo, Japan), and ammonia levels (WAK-NH4-4, Kyoritsu chemical lab, Tokyo, Japan) during the feeding trial. Fish larvae were divided into two groups for two individual feeding trials. A randomized design was applied to eliminate any possible external environmental factors during the study. For the first stage of the study, 125 fish larvae (0.19 ± 0.05 g) were divided into five groups, receiving lyophilized microalgae powder with fish oil and eel powder as a carrier for 70 days. In the second feeding trial, 40 fish (0.28 ± 0.06 g) were selected for investigating the extracted astaxanthin diets.

2.2. Microalgae Preparation. H. pluvialis, preserved in Prof. MC Lee's laboratory at NTOU, was applied in this study with a Jaworski's medium (J.M.) modified from Rushan et al. [25] using a commercial fertilizer (Hyponex no. 4, Hyponex, Marysville, OH, USA) to meet the nutrient requirements. The culture of *H. pluvialis* followed the process described by Park et al. [26]. In brief, the microalgae cell was inoculated in a 20-L serum bottle supplied with a modified J.*M. medium* and subjected to 50 μmol m^{-2} s^{-1} of lighting with a density of $1.59 \pm 0.37 \times 10^4$ cells/mL before enrichment. To increase the astaxanthin production yields further, vegetable cells were assigned to an astaxanthin enrichment process. After exposure under light with a density of 130 μmol m^{-2} s^{-1} at 28°C for 24 hr, the red cyst cells of *H. pluvialis* were harvested for further study [26, 27].

2.3. Astaxanthin Extraction. After the generation of astaxanthin-rich *H. pluvialis*, the cells were collected by centrifugation at 5,000 rpm for 10 min (Himac CR21G, Hitachi, Japan). After the supernatant was removed, the cells were freeze-dried (FDU-1200, EYELa, Bohemia, NY, USA) with varying concentrations. To achieve the best extraction quality, chlorophyll was removed before the extraction, following the method described by Bubrick [17]. Briefly, 0.01 g of the dried *H. pluvialis* powder was added to 5 mL of 30% methanol (Sigma Aldrich, St. Louis, MO, USA) with 5% KOH (Sigma Aldrich) added in. The mixture was incubated under 70°C for 5 min to lyse the chlorophyll. The product was subjected to centrifugation at 3,750 rpm for 10 min. After the supernatant was removed, the product was stored in RT for further processing.

To investigate the extraction efficiency, two extraction methods, acetic acid-DMSO and HCl–acetone, were applied with some modifications in this study. The acetic acid-DMSO method was modified from Bubrick [17]. Chlorophyll was removed by combining the cell powder, 100 μL of acetic acid, and 3 mL of DMSO (Sigma Aldrich) solvent. The mixture was heated at 70°C in a water bath for 5 min before the supernatant was collected via centrifugation of the product at 3,750 rpm for 10 min. This process was repeated five times to extract astaxanthin fully from the samples until the samples were colorless. The product was measured by comparing its absorbance at 480 nm with that of the commercial standard astaxanthin on a spectrophotometer (Genesys 30, Thermo Scientific, Waltham, MA, USA).

The HCl–acetone method followed the process of Sarada et al. [28]. As in the acetic acid-DMSO method, chlorophyll was removed by adding the cell powder to 5 mL of 4N HCl (Sigma Aldrich) and heated in a water bath at 70°C for 5 min to lyse the cell. Afterward, the cell debris was collected by centrifugation at 3,750 rpm for 10 min; the pellet was washed with deionized water to balance the pH before homogenizing the product. For acetone extraction, 10 mL of acetone was applied to the sample and incubated at room temperature for

Table 1: Ingredient compositions of the experimental diets for *A. ocellaris*.

Ingredient	Control	AH50	H100/AH100	H200/AH200	H400	As100	As200
Eel meal[a]	91.0	91.0	91.00	91.00	91.00	91.00	91.00
Fish oil	9.00	9.00	9.00	9.00	9.00	9.00	9.00
H. pluvialis powder[b]	0.00	0.24	0.50	0.97	1.93	0.00	0.00
Synthetic astaxanthin[c]	0.00	0.00	0.00	0.00	0.00	0.10	0.20

[a]Commercial eel diet (TAIROUN, Taiwan). [b]*H. pluvialis* powder: astaxanthin (2.07%). [c]Synthetic astaxanthin: astaxanthin (10%).

1 hr (light avid). After evaporation, the absorbance of the extracted astaxanthin was measured and compared with that of the commercial standard astaxanthin (Carophyll Pink, Nice Garden Industrial, Taipei, Taiwan) [29, 30].

2.4. Experimental Diet Preparation. To satisfy the study goals, two diets were prepared to examine the effects. In Diet I, a commercial eel diet (Tairoun, Kaohsiung, Taiwan) was applied in this study as the major ingredient. Freeze-dried *H. pluvialis* powder with gradient concentrations of 0 (Control), 100 (H100), 200 (H200), and 400 mg/kg (H400) were premixed with 9% fish oil (Hong sheng, Taipei, Taiwan) as a carrier. The diets were air-dried and preserved in the fridge before use. To investigate the effects of *H. pluvialis*-extracted astaxanthin further, HCl–acetone-extracted astaxanthin was used in Diet II. Once again, eel powder was added to varying concentrations of freeze-dried *H. pluvialis* powder: 0 (control), 50 (AH50), 100 (AH100), 200 (AH200), and 400 mg/kg (AH400), supplied with 9% fish oil. Diets I and II were compared with the commercially synthesized astaxanthin with concentrations of 100 (As100) or 200 mg/kg (As200) as a positive control. The ingredient formulation is shown in Table 1.

2.5. Digital Image Acquisition and Processing (DIAP). The changes in skin color between the trial and control individuals were compared using the DIAP technique. Images were captured using the Compact Digital Camera (COOLPIX P310, Nikon, Japan) under stable light conditions and processed using Adobe® Photoshop® CS6c for color scale calibration before sampling. Color saturation and brightness were identified using CIE 1976 (L*, a*, and b*) and CIELAB color space to convert images to L* (light), a* (red signal), b* (yellow signal), and H* (hue) values. For the sampling, several points on the fish bodies were zoomed in for analysis: these points were the center of the upper and lower edges from the first to the second white band and the center of the second white band to the final white band (defined as the band closest to the tail).

2.6. Statistics Analysis. The results of the coloration index were analyzed by using Statistical Analysis System (SAS 9.4, USA) software. The normality of all datasets was tested using Shapiro–Wilk tests, with $p < 0.05$ observed before being subjected to a one-way analysis of variance (ANOVA), followed by Duncan's new-multiple range tests for a post hoc analysis in a 95% confidence region. The significant differences within the treatment were represented as various letters.

Table 2: The astaxanthin extraction yields from *H. pluvialis* in different treatments.

Treatment	Astaxanthin yield (mg/g cell)	Astaxanthin yield (mg/g cell) after chlorophyll is removed
Acetic acid-DMSO	$19.20 \pm 0.49^{B,a}$	$15.80 \pm 0.98^{B,b}$
HCl–acetone	$21.99 \pm 0.52^{A,a}$	$20.41 \pm 0.52^{A,a}$

Data are means ± SD, $n = 5$. Means in the same column with different letters (A and B) are significantly different ($p < 0.05$). Means in the same line with different letters (a and b) are significantly different ($p < 0.05$).

3. Results

3.1. Comparison of the Extraction Efficacy of the Acetic Acetic-DMSO and HCl–Acetone Methods. Two chemical-based extraction methods were assigned to the lyophilized *H. pluvialis* red cyst cells. The maximal yield of astaxanthin under the HCl–acetone extraction method was 21.99 ± 0.52 mg/g per cell, while that under the acetic acid-DMSO method was 19.20 ± 0.49 mg/g of astaxanthin per cell (Table 2). A significant increase in product amount was found with $p < 0.05$, suggesting that the HCl–acetone method had a better astaxanthin extraction efficiency from the *H. pluvialis* red cyst cells. The extraction products were preserved in a freezer and applied for the second stage of functional testing on clownfish.

3.2. Stage 1 Feeding Trial: Impacts of Astaxanthin-Enriched Lyophilized H. pluvialis Powder as Dietary Supplement on the Skin Coloration Performance of Clownfish. In stage one, 125 clownfish with an average body weight of 0.2 ± 0.01 g were divided into five groups, with 25 fish per treatment for a 70-day accumulation. Gradient dosages of freeze-dried *H. pluvialis* powder were applied in this study and compared with the commercially synthesized astaxanthin as the positive control.

The coloration performance was observed biweekly, and a significant increase in the red color signal (a*) was observed from Week 2 and onwards in the group that received the positive control (As200), while the hue (H*) decreased. Interestingly, with the supply of 200 mg/kg of algae powder, the signal for yellow color (b*) was significantly increased after continuous feeding for 4 weeks, with the red color increasing after 6 weeks (Table 3, Figure 1). A trend was found in the result: the accumulation of red color in the epidermis was primarily derived from astaxanthin, and the enriched algae powder could improve yellow pigmentation as a side effect.

Table 3: The effect of *H. pluvialis* algae powder on *A. ocellaris* skin color parameters (a*, b*, and H* values).

a*	Treatment	Week					
		0	2	4	6	8	10
—	Control	$40.00 \pm 4.99^{A,ab}$	$36.13 \pm 5.40^{B,abc}$	$41.13 \pm 6.37^{B,a}$	$33.20 \pm 6.70^{D,c}$	$35.47 \pm 5.20^{C,abc}$	$34.13 \pm 7.70^{B,bc}$
—	H100	$40.67 \pm 3.53^{A,ab}$	$37.33 \pm 3.49^{B,bc}$	$42.20 \pm 5.64^{B,a}$	$36.63 \pm 6.43^{CD,bc}$	$37.43 \pm 3.68^{C,bc}$	$34.80 \pm 5.36^{B,c}$
—	H200	$41.73 \pm 8.50^{A,a}$	$37.27 \pm 6.19^{B,a}$	$39.87 \pm 6.80^{B,a}$	$39.00 \pm 6.39^{BC,a}$	$37.37 \pm 6.23^{C,a}$	$39.00 \pm 7.94^{B,a}$
—	H400	$42.87 \pm 6.38^{A,a}$	$40.93 \pm 5.53^{B,a}$	$43.80 \pm 6.05^{B,a}$	$42.67 \pm 6.05^{B,a}$	$43.07 \pm 5.86^{B,a}$	$39.97 \pm 6.54^{B,a}$
—	As200	$41.50 \pm 5.69^{A,d}$	$46.93 \pm 3.51^{A,c}$	$53.43 \pm 4.06^{A,ab}$	$53.00 \pm 2.41^{A,ab}$	$51.63 \pm 5.43^{A,b}$	$55.93 \pm 4.01^{A,a}$
b*	Treatment	Week					
		0	2	4	6	8	10
—	Control	$69.20 \pm 3.40^{A,a}$	$66.50 \pm 4.11^{A,ab}$	$66.40 \pm 6.68^{AB,ab}$	$63.43 \pm 4.94^{AB,bc}$	$62.30 \pm 3.78^{A,bc}$	$61.70 \pm 4.52^{A,c}$
—	H100	$68.67 \pm 3.18^{A,a}$	$66.67 \pm 3.31^{A,ab}$	$65.43 \pm 2.29^{AB,b}$	$63.57 \pm 3.69^{AB,bc}$	$63.63 \pm 2.12^{A,bc}$	$61.43 \pm 4.71^{A,c}$
—	H200	$70.77 \pm 2.89^{A,a}$	$68.10 \pm 7.41^{A,ab}$	$68.23 \pm 3.52^{A,ab}$	$66.00 \pm 4.66^{A,abc}$	$64.30 \pm 6.30^{A,bc}$	$61.33 \pm 3.51^{A,c}$
—	H400	$70.30 \pm 2.25^{A,a}$	$67.40 \pm 3.65^{A,ab}$	$63.50 \pm 6.28^{B,bc}$	$64.50 \pm 3.75^{A,bc}$	$62.53 \pm 4.32^{A,c}$	$55.53 \pm 5.78^{B,d}$
—	As200	$70.27 \pm 2.15^{A,a}$	$65.43 \pm 3.61^{A,b}$	$62.83 \pm 2.75^{B,bc}$	$60.53 \pm 2.66^{B,c}$	$60.57 \pm 4.42^{A,c}$	$59.93 \pm 3.55^{A,c}$
H*	Treatment	Week					
		0	2	4	6	8	10
—	Control	$60.00 \pm 3.49^{A,ab}$	$61.45 \pm 4.72^{A,ab}$	$58.25 \pm 3.86^{AB,b}$	$62.54 \pm 4.35^{A,a}$	$60.46 \pm 3.03^{A,ab}$	$61.38 \pm 4.58^{A,ab}$
—	H100	$59.35 \pm 2.93^{A,ab}$	$60.76 \pm 2.56^{A,a}$	$57.27 \pm 3.80^{AB,b}$	$60.19 \pm 3.85^{A,ab}$	$59.56 \pm 2.76^{A,ab}$	$60.59 \pm 2.91^{AB,a}$
—	H200	$59.67 \pm 5.37^{A,a}$	$61.22 \pm 4.63^{A,a}$	$59.78 \pm 4.94^{A,a}$	$59.51 \pm 4.11^{AB,a}$	$59.90 \pm 3.78^{A,a}$	$57.84 \pm 4.64^{B,a}$
—	H400	$58.72 \pm 4.23^{A,a}$	$58.80 \pm 3.30^{A,a}$	$55.46 \pm 2.85^{B,b}$	$56.64 \pm 3.15^{B,ab}$	$55.57 \pm 2.72^{B,b}$	$54.41 \pm 2.45^{C,b}$
—	As200	$59.51 \pm 3.86^{A,a}$	$54.35 \pm 2.44^{B,b}$	$49.67 \pm 1.40^{C,c}$	$48.79 \pm 1.18^{C,cd}$	$49.61 \pm 2.56^{C,c}$	$46.99 \pm 2.23^{D,d}$

Data are means ± SD, $n = 5$. a* represents red. b* represents yellow. H* represents hue. Means in the same column with different letters (A, B, C, and D) are significantly different ($p < 0.05$). Means in the same line with different letters (a, b, c, and d) are significantly different ($p < 0.05$).

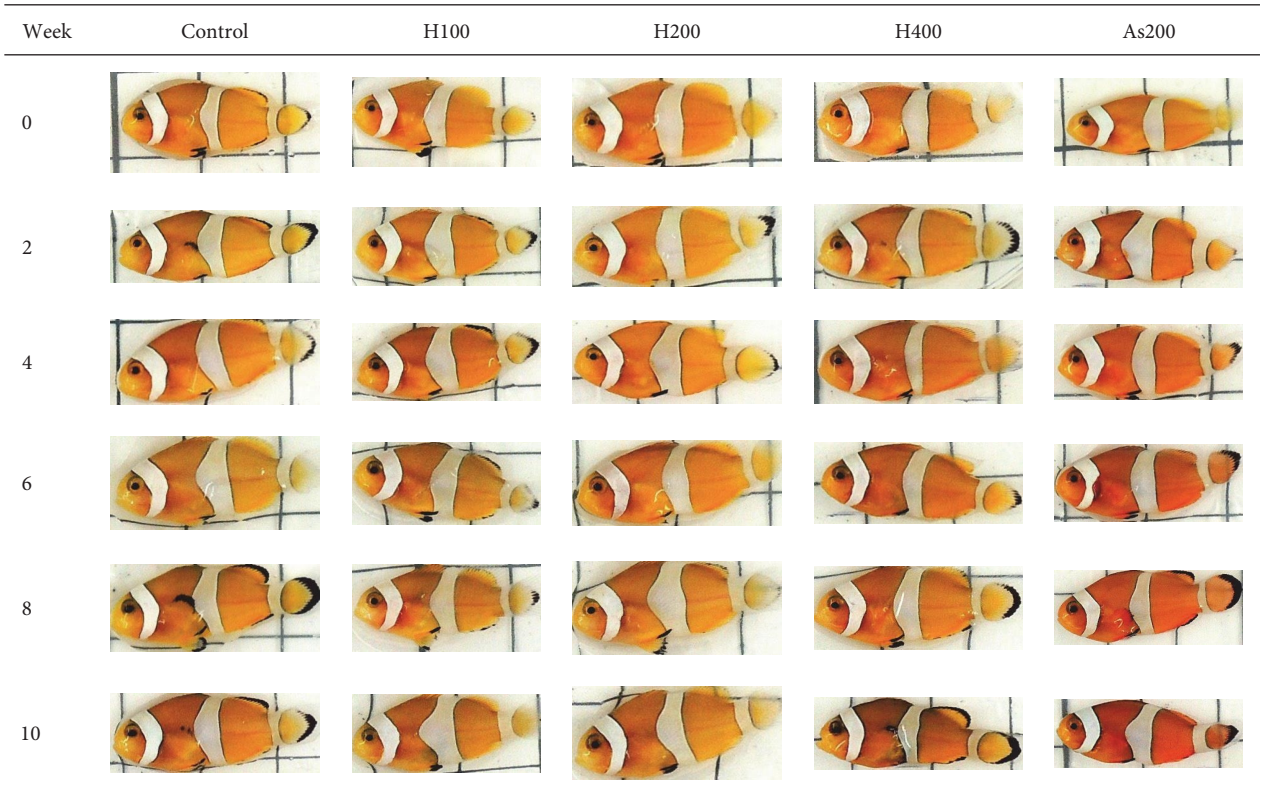

Figure 1: Pigmentation of *A. ocellaris* that were fed diets containing 100, 200, and 400 mg/kg of *H. pluvialis* algae powder (H100, H200, H400, respectively) for 10 weeks, compared with those fed with synthetic astaxanthin (200 mg/kg, As200), in the first stage of the study.

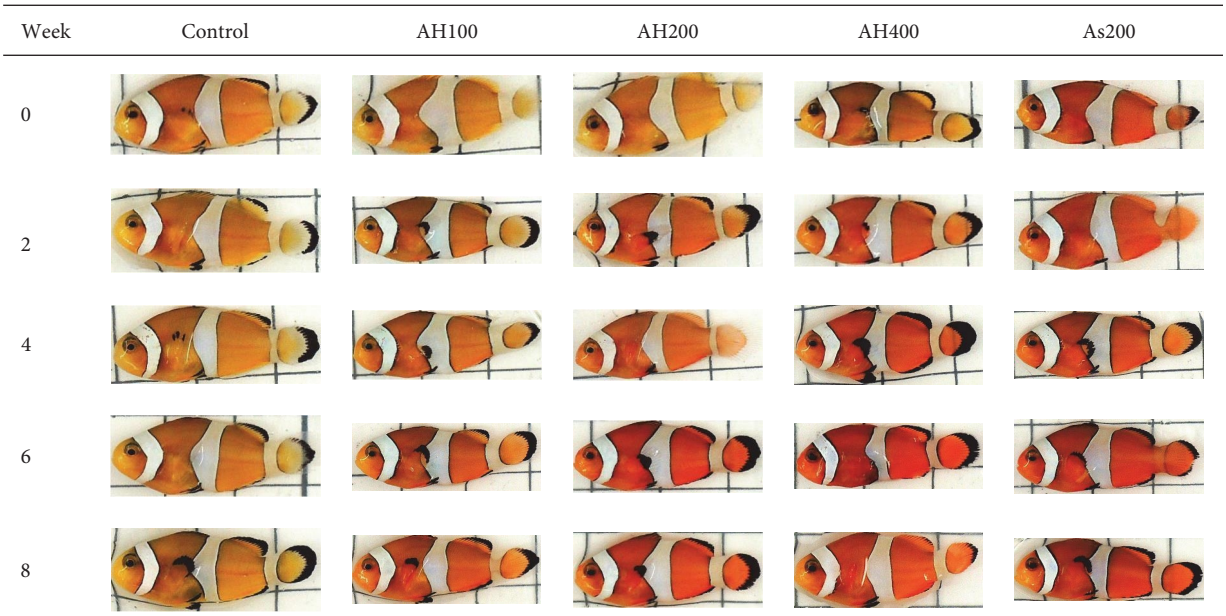

FIGURE 2: Pigmentation of *A. ocellaris* that were fed diets containing 100, 200, and 400 mg/kg of astaxanthin extracted from *H. pluvialis* (AH100, AH200, AH400, respectively) for 8 weeks, compared with those fed with synthetic astaxanthin (200 mg/kg, As200), in the second feeding trial.

3.3. Stage 2 Feeding Trial: The Effect of HCl–Acetone-Extracted Astaxanthin as a Meal Additive on the Enhancement of Clownfish Pigmentation. In stage two, the effects of extracted astaxanthin on clownfish were investigated. Fish larvae with an average body weight (0.28 ± 0.01 g) was assigned to the study. A 56-day pigmentation accumulation was conducted with 40 fish, which were divided into five treatments of eight fish each. The changes in color performance are shown in Figure 2. The results showed that compared to the synthesized product, the HCl–acetone-extracted astaxanthin demonstrated a similar effect, primarily increasing the red signal in the epidermis while decreasing the yellow signal in the meantime. The fish received over 200 mg/kg of astaxanthin (AH200, AH400, and As200), showing a significant increase in the red signal from week six. Notably, the extracted astaxanthin led to no significant difference in the red coloration promotion under the same applied concentrations (AH200 and As200). Moreover, the yellow signal at week eight significantly increased in the clownfish fed with astaxanthin from algae sources (AH200) when compared with that in the clownfish fed with synthesized astaxanthin (As200). To sum up, after being orally treated with HCl–acetone-extracted astaxanthin, the clownfish exhibited increased red color performance and similar effects to those treated with synthesized products (Table 4 and Figure 3).

4. Discussion

Astaxanthin is a food coloring and feed additive that is commonly used to enhance pigmentation performance in salmon and trout [31, 32]. It is given to maintain the market value of the meat fish and also aiding in oxidative regulation, promoting fish adaptation and recovery from stress responses [33, 34]. With the benefits of mass production at lower costs, the artificially synthesized process is the majority approach for providing a stable supply of astaxanthin and satisfying both aquaculture and nutraceutical purposes [35]. Nevertheless, the recent increase in astaxanthin demand and price has pushed aquaculture farmers out of the accession [36]. An astaxanthin production process with higher efficiency that is cheaper in price can provide an alternative way of obtaining astaxanthin. Microalgae, *H. pluvialis*, has been regarded in the industry as the primary freshwater astaxanthin producer [29]. Recently, with the control of carbon dioxide emissions becoming stricter, the culture of algae as a tool in carbon trading has garnered attention [37]. The production of algae for carbon trading further grants the generation of related algae byproducts as a new industry model.

Previous studies have investigated the utilization of *H. pluvialis* as a coloring agent for rainbow trout (*Oncorhynchus mykiss*) and red devil fish (*Cichlasoma citrinellum*). Results have revealed that without any pretreatment, algae powder (40 mg/kg for rainbow trout and 400 mg/kg for red devil fish) can only provide a small but significant pigment deposition improvement in rainbow trout and that algae cell wall is a barrier for pigment utilization in fish [38, 39]. Interestingly, compared to the vertebrates, invertebrates seem to yield better results with the use of *H. pluvialis* powder. By consuming 200 mg/kg of *H. pluvialis* powder in their diet, Chinese mitten crab (*Eriocheir sinensis*) can significanly improve their skin color performance [40]. Another study revealed that the utilization of algae is strongly affected by algae conformation and the animal's digestibility [41].

In this study, *H. pluvialis* powder was applied to clownfish in the first stage of the feeding trial. Results showed that the cell wall of the *H. pluvialis* cyst may be a barrier to astaxanthin utilization. As the mature *H. pluvialis* red cyst

Table 4: The effect of astaxanthin extracted from *H. pluvialis* on *A. ocellaris* skin color parameters (a*, b*, and H* values).

a*	Treatment	Week				
		0	2	4	6	8
—	Control	$34.13 \pm 7.70^{B,a}$	$34.00 \pm 5.63^{B,a}$	$32.40 \pm 5.06^{D,a}$	$34.90 \pm 5.55^{C,a}$	$31.50 \pm 6.21^{C,a}$
—	AH100	$34.80 \pm 5.36^{B,c}$	$37.90 \pm 5.28^{B,bc}$	$41.00 \pm 6.29^{C,b}$	$48.80 \pm 2.79^{B,a}$	$49.97 \pm 6.90^{B,a}$
—	AH200	$39.00 \pm 7.94^{B,c}$	$51.33 \pm 6.33^{A,b}$	$51.03 \pm 3.47^{B,b}$	$58.90 \pm 3.53^{A,a}$	$58.57 \pm 4.20^{A,a}$
—	AH400	$39.97 \pm 6.54^{B,c}$	$51.20 \pm 5.97^{A,b}$	$56.67 \pm 4.05^{A,a}$	$58.73 \pm 4.84^{A,a}$	$58.67 \pm 3.94^{A,a}$
—	As200	$55.93 \pm 4.01^{A,a}$	$56.10 \pm 3.89^{A,a}$	$54.73 \pm 5.09^{AB,a}$	$55.80 \pm 3.76^{A,a}$	$53.57 \pm 5.13^{AB,a}$
b*	Treatment	Week				
		0	2	4	6	8
—	Control	$61.70 \pm 4.52^{A,a}$	$58.20 \pm 2.44^{A,ab}$	$61.10 \pm 2.68^{A,ab}$	$60.67 \pm 4.18^{A,ab}$	$57.40 \pm 5.47^{AB,b}$
—	AH100	$61.43 \pm 4.71^{A,a}$	$54.97 \pm 4.38^{B,b}$	$56.30 \pm 3.34^{B,b}$	$57.00 \pm 2.67^{AB,b}$	$58.87 \pm 4.80^{AB,ab}$
—	AH200	$61.33 \pm 3.51^{A,a}$	$60.00 \pm 3.95^{A,a}$	$59.43 \pm 4.23^{AB,a}$	$58.20 \pm 3.46^{AB,a}$	$59.93 \pm 2.60^{A,a}$
—	AH400	$55.53 \pm 5.78^{B,a}$	$58.40 \pm 2.60^{A,a}$	$58.10 \pm 2.57^{AB,a}$	$55.90 \pm 4.33^{B,a}$	$56.37 \pm 2.74^{AB,a}$
—	As200	$59.93 \pm 3.55^{A,a}$	$59.83 \pm 3.17^{A,a}$	$56.87 \pm 5.04^{B,ab}$	$55.70 \pm 4.73^{B,ab}$	$55.30 \pm 5.90^{B,b}$
H*	Treatment	Week				
		0	2	4	6	8
—	Control	$61.38 \pm 4.58^{A,a}$	$59.88 \pm 3.62^{A,a}$	$62.19 \pm 3.31^{A,a}$	$60.21 \pm 3.42^{A,a}$	$61.41 \pm 3.92^{A,a}$
—	AH100	$60.59 \pm 2.91^{AB,a}$	$55.55 \pm 2.17^{B,b}$	$54.13 \pm 3.49^{B,b}$	$49.44 \pm 1.48^{B,c}$	$49.80 \pm 3.19^{B,c}$
—	AH200	$57.84 \pm 4.64^{B,a}$	$49.57 \pm 2.95^{C,b}$	$49.33 \pm 2.14^{C,b}$	$44.66 \pm 1.46^{C,c}$	$45.70 \pm 2.12^{C,c}$
—	AH400	$54.41 \pm 2.45^{C,a}$	$48.90 \pm 2.73^{CD,b}$	$45.75 \pm 2.03^{D,c}$	$43.59 \pm 1.56^{C,cd}$	$43.88 \pm 1.40^{C,d}$
—	As200	$46.99 \pm 2.23^{D,a}$	$46.87 \pm 2.04^{D,ab}$	$46.11 \pm 1.90^{D,ab}$	$44.92 \pm 1.74^{C,b}$	$45.88 \pm 2.08^{C,ab}$

Data are means ± SD, $n = 5$. a* represents red. b* represents yellow. H* represents hue. Means in the same column with different letters (A, B, C, and D) are significantly different ($p < 0.05$). Means in the same line with different letters (a, b, c, and d) are significantly different ($p < 0.05$).

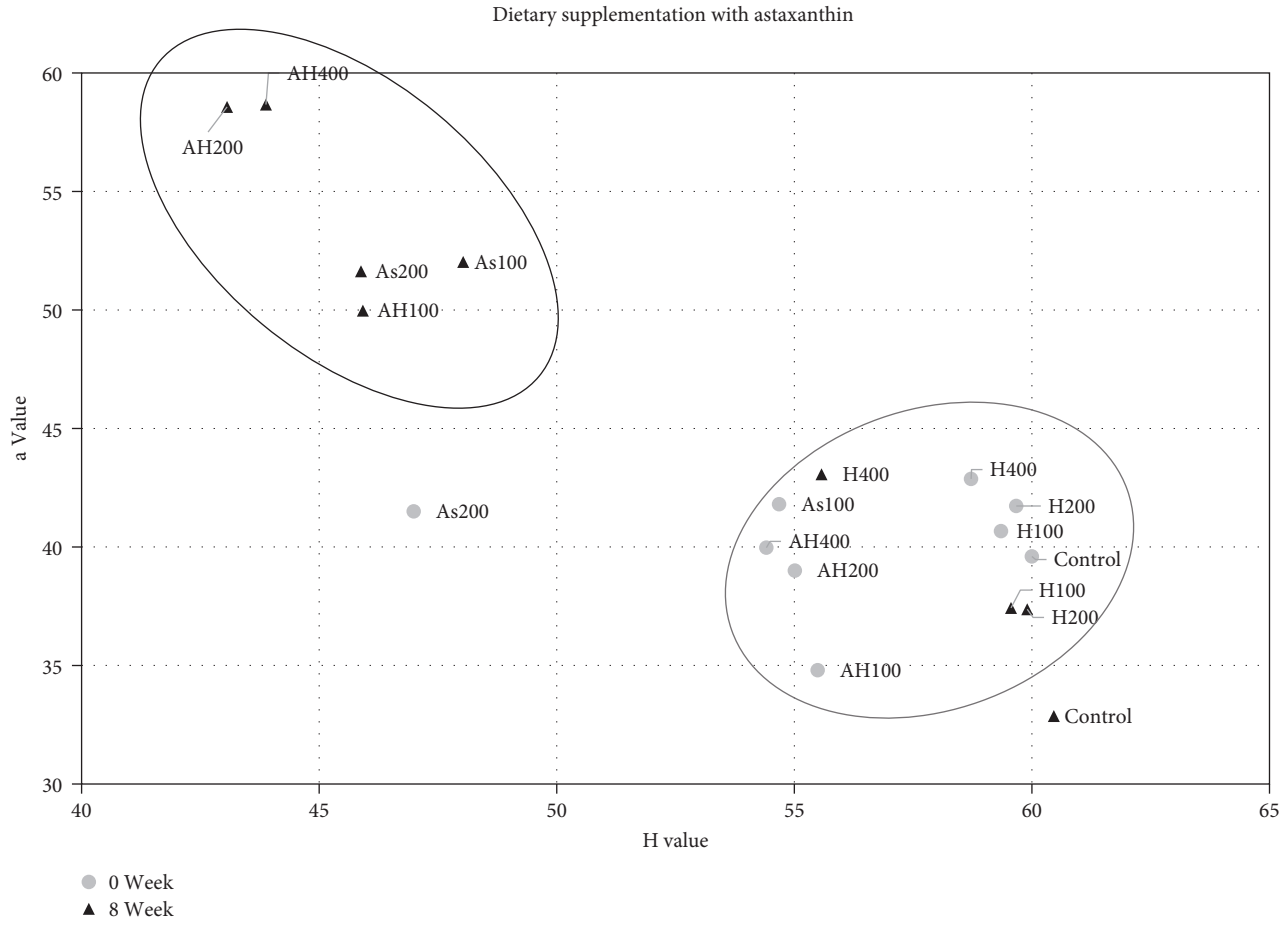

Figure 3: Changes in the a* and H* color parameter values of *A. ocellaris* that were fed diets supplemented with *H. pluvialis* algae powder or extracted astaxanthin for eight weeks. H* represents hue, a* represents red. Circle indicates the trend of change in coloration, carried out by the dietary supplementation. Diet: control, diet without added pigment; H, *H. pluvialis* algae powder; AH, astaxanthin extracted from *H. pluvialis*; As, synthetic astaxanthin.

contains a high abundance of mannose and cellulose, it is robust in maintaining stability in an acidic environment and is not digestible by most aquatic animals [42–44]. A recent study revealed that the absorption of astaxanthin is affected by the intestinal microbiome, further suggesting that astaxanthin derived from *H. pluvialis* has the greatest utilization rate compared to other carotenoids [45, 46]. This supports our finding that changes in skin coloration (red signal) performance are mainly sourced from the astaxanthin of *H. pluvialis*.

To maximize the production value of astaxanthin from microalgae, the extraction methods were investigated. After altering environmental factors to induced astaxanthin accumulation, the cell lysis required an additional process before purification. Currently, the commercial production process, which uses both chemical and physical methods followed by a supercritical CO_2 treatment to extract the product, achieves high-production yields but is expensive [47]. Instead of supercritical extraction, two chemical-based methods were used in this study in an affordable and practical way. The HCl–acetone method showed a significant improvement in efficiency (77.41% rise in production yields) compared to the acetic acid-DMSO method after purification. The same result was found in Sarada et al. [28], which supported the claim that adding 2N HCl as a lysis reagent can increase the astaxanthin extraction from *H. pluvialis*.

Finally, HCl–acetone-extracted astaxanthin was blended with eel powder to examine its effects on coloration performance while ensuring that the products remained functional. Out of all other treatments, an oral administration of 200 and 400 mg/kg of HCl–acetone-extracted astaxanthin for 4 weeks led to the clownfish achieving the best red color performance. Similar results of a significant increase in pigmentation performance can also be found in red porgy (*Pagrus pagrus*) and rose barb (*Puntius conchonius*) after receiving 50–80 mg/kg of astaxanthin [48, 49]. Using synthetic pigments and microalgae powder as additives in meals has generally led to no changes in yellow pigment performance in the short term but has shown a tendency to increase yellow pigment performance under high doses and longer periods. To sum up, astaxanthin as a diet additive could improve pigmentation performance in fish. Astaxanthin can enhance the vibrancy of the coloration of clownfish (*A. ocellaris*). However, the effect of increasing their coloration can be further enhanced by first extracting astaxanthin from *H. pluvialis* microalgae and then incorporating it into their diet.

5. Conclusions

In this study, the effects of *H. pluvialis*-derived astaxanthin and artificially synthesized astaxanthin products as coloration enhancers on clownfish (*A. ocellaris*) were evaluated and compared. The HCl–acetone extraction method had a higher productivity, and the removal of chlorophyll did not affect the average product mass. Furthermore, the extracted astaxanthin in this study significantly increased the color performance of clownfish, especially the 200 mg/kg-dose group. Finally, the lyophilized *H. pluvialis* powder diets administrated for 10 weeks did not promote skin coloration performance, which indicated that clownfish may not have enough digestibility of the *H. pluvialis* cyst cell to achieve the ideal color from an algae diet.

In conclusion, astaxanthin extracted from the *H. pluvialis* cyst cell by using the HCl–acetone method can significantly enhance the pigmentation of clownfish, especially their red color performance after 8 weeks of a feeding trial. The results of this study showed that the use of astaxanthin as a dietary supplement could be a dependable and practical way for improving the pigmentation performance of ornamental fish.

Data Availability

Data supporting this research article are available on reasonable request.

Ethical Approval

All procedures in this study were conducted in accordance with the Institutional Animal Care and Use Committee's (Approval No.: 105033) approved protocols.

Disclosure

This study is a part of the thesis "Effect of Dietary Supplementation with *Haematococcus pluvialis* on Survival, Growth, and Pigmentation of *Amphiprion ocellaris*" submitted by Chia-Yu Chang to the National Taiwan Ocean University for the degree of master in aquaculture.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Meng-Chou Lee contributed in the conceptualization and methodology. Chih-Yang Huang, Fan-Hua Nan, and Po-Tsang Lee contributed in the methodology and resources. Jing Huang contributed in the investigation and formal analysis. Chia-Yu Chang contributed in the investigation, formal analysis, and writing—original draft. Fan-Hua Nan contributed in the writing—review and editing. Meng-Chou Lee and Chih-Yang Huang are the co-first authors.

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