Nutritional Study of Dietary Astaxanthin on Performances of Kuruma Shrimp *Marsupenaeus japonicus*

(クルマエビにおける飼料性アスタキサンチンの栄養学的研究)

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Contents

Abstract

The color of kuruma shrimp flesh is an important criterion of flesh quality and affects consumer choice thus determines its price in the Japanese market. Since cultured shrimp are less colorful than the wild shrimp, a necessary to provide a dietary pigment source in the diet is considered. Astaxanthin (Ax) is a pigment producing a red color for many aquatic animals was certified as one of the most useful pigment for shrimp. To provide the proper rations of Ax, in aqua-feed, it is necessary to reveal the optimum supplemental amounts and the interactions between Ax and other additives.

First part of the studies were aimed to determine the optimum supplemental amounts of chemically synthesized astaxanthin (Ax) in larval, post-larval, and juvenile kuruma shrimp. An 8-day and a 30-day feeding trials were conducted on larval and post-larval kuruma shrimp, respectively. Six dietary levels of Ax (0, 50, 100, 200, 400, and 800 mg kg^{-1} diet) were added to a baseline diet. Broken-line regression analysis indicated that the optimal levels for growth and stress resistance of larvae were 168.9 mg kg^{-1} and 82.1 mg kg^{-1} diet, respectively. Broken-line regression analysis indicated that the optimal levels for growth and stress resistance of post-larvae were 108.7 mg kg^{-1} and 178.1 mg kg^{-1} diet, respectively. Then a 56-day feeding trial was conducted on juvenile kuruma shrimp. Six dietary levels of Ax (0, 200, 400, 800, 1200, and 1600 mg kg^{-1} diet) were added to a baseline diet. Broken-line regression analysis indicated that the optimal levels for growth, immune responses, and pigmentation were 401, 420, and 404 mg kg^{-1} diet, respectively.

Second part of the studies were conducted to evaluate the interaction of vitamin E (α-Toc) and cholesterol (CHO) with Ax, respectively. Six experimental diets containing 2 levels of Ax (0 and 0.6 g kg^{-1} diet) and 3 levels of CHO (0, 6, and 20 g kg^{-1} diet) were formulated in 2 × 3 factorial design. The results showed that interactive effects by dietary CHO and Ax existed on growth parameters, lipase activity, pigmentation, and total Ax content in different parts of shrimp body. The addition of CHO can enhance the positive effects of dietary Ax. Then, a 2 × 3 factorial experiment was conducted with six experimental diets containing two levels of Ax (0 and 0.6 g kg^{-1} diet) and three levels of α-Toc (0, 0.2, and 1 g kg^{-1} diet). The results showed that dietary Ax and α-Toc functioned interactively on growth performance and Ax content in kuruma shrimp.

Overall, carefully dosed Ax supplementation is a beneficial nutritional strategy for kuruma shrimp. Dietary Ax with α-Toc and cholesterol functioned interactively. My study fills a data gap regarding the interactive effects of these two supplements with Ax and provides practical information to improve pigmentation and performance of kuruma shrimp, thus enhancing their commercial value. It is very important to consider about the proper Ax ratios in aqua-feed. Further studies need to be contributed to reveal the mechanisms of related additives interactions in kuruma shrimp.

Abstract (Japanese)

クルマエビの市場価格を決定する要因の一つが、可食部の美しい赤色である。クルマエビ養殖飼料は、魚粉やイカ粉末を主原料とするため、養殖クルマエビは天然クルマエビより色調が劣るといわれている。そのため、養殖飼料に飼料性色素を添加し、色調を改善することが必要である。アスタキサンチン（Ax）は一般的な飼料色素源として、エビの赤色を深めるのには効果が高い色素である。しかし、クルマエビ幼生と稚エビにおける Ax 至適添加量については知見が少なく、他の飼料栄養素との交互作用についても検討例は少ない。本研究は、幼生期と稚エビ期の Ax 添加量について、成長、免疫応答及び色調などの指標を用いて検討した。

実験１：Ax を８％含有する製剤（Carophyll Pink）の添加量を変えた５種類の微粒子飼料（0, 50, 100, 200, 400, 800 mg kg-1 diet）をゾエア１期の幼生に給餌し、ポストラーバ１期までの８日間飼育した。broken-line 解析により、増重を指標した場合は 168.9 mg kg-1 飼料、淡水ストレスに対する耐性では、82.1mg kg-1 飼料が至適添加量であることがわかった。

実験２：Ax 添加量の異なる５種類の微粒子飼料（0, 50, 100, 200, 400, 800 mg kg-1 diet）をポストラーバ 15 期から 30 日間飼育した。飼育試験の結果、増重では、108.7 mg kg-1 飼料、淡水ストレスに対する耐性では、178.1 mg kg-1 飼料が至適添加量であることを明らかにした。

実験３：Ax 添加量の異なる５種類の配合飼料（0, 200, 400, 800, 1200, 1600 mg kg-1 diet）を稚エビに給餌し、56 日間飼育した. 増重、免疫指標、色調を基にした broken-line 解析により、401 mg kg-1、420 mg kg-1、404 mg kg-1 飼料がそれぞれの至適添加量であることがわかった。

実験 4：クルマエビの必須栄養素であるコレステロールと Ax の交互作用を検討した。2 段階の Ax 添加量（0 及び 0.6 g kg-1 diet）と 3 段階のコレステロール添加量（0, 6, and 20 g kg-1 diet）を用いて試験飼料を作製し飼育実験を行った。飼育試験の結果によって、Ax 及び CHO は、増重、免疫指標、色調いずれの指標でも交互作用は確認された。

実験 5：クルマエビの必須栄養素であり、抗酸化効果が報告されているビタミン E と Ax の交互作用を検討した。2 段階の Ax 添加量（0 及び 0.6 g kg-1 diet）と 3 段階のビタミン E 添加量（0, 0.2, and 1 g kg-1 diet）を用いて試験飼料を作製し飼育実験を行った。飼育試験の結果によって、Ax 及び α-Toc は、いずれの指標でも交互作用は確認された。

本研究の結果、飼料性アスタキサンチンは幼生期のクルマエビの成長と環境ストレス耐性を改善し、稚エビ期では成長とともに免疫応答及び色調を改善することが明らかになった。

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Publications in Peer Reviewed Journals

1. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M.A.O., Zhang, Y.K., 2018. Effects of Dietary Astaxanthin Supplementation on Survival, Growth and Stress Resistance in Larval and Post-larval Kuruma Shrimp, *Marsupenaeus japonicus*. Aquaculture Research. 49:2225-2232.
2. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., Hossain, M.S., Moss, A.S., 2018. Effects of Dietary Astaxanthin Supplementation on Juvenile Kuruma Shrimp, *Marsupenaeus japonicus*. Aquaculture. 491:197-204.
3. Dawood, M.A.O., Koshio, S., Ishikawa, M., EI-Sabagh, M., Yokoyama, S., **Wang, W.L**., Olivier, A., 2017. Physiological response, blood chemistry profile and mucus secretion of red sea bream (*Pagrus major*) fed diets supplemented with *Lactobacillus rhamnosus* under low salinity stress. Fish Physiology and Biochemistry. 43:179-192.
4. Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., Dossou, S., **Wang, W.L**., 2018. Influence of dietary inosine and vitamin C supplementation on growth, blood chemistry, oxidative stress, innate and adaptive immune responses of red sea bream, *Pagrus major* juvenile. Fish & Shellfish Immunology. 82: 92-100.

Publications in Contribution

1. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M.A.O., Hossain, M.S. Interactive effects of dietary Astaxanthin supplementation and vitamin E on performance of kuruma shrimp, *Marsupenaeus japonicus*. Aquaculture (Under review).
2. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M.A.O., Hossain, M.S., Zaineldin, A.I. Interactive effects of dietary Astaxanthin supplementation and cholesterol on performance of kuruma shrimp, *Marsupenaeus japonicus*. Aquaculture (Under review).

3. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M.A.O., Hossain, M.S., Zaineldin, A.I. Interactive effects of dietary Astaxanthin supplementation and vitamin C on performance of kuruma shrimp, *Marsupenaeus japonicus*. Fish and Shellfish Immunology (Under review).

Conference Abstracts

1. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., 2018. Interactive effects of dietary astaxanthin and cholesterol on the performances of kuruma shrimp, *Marsupenaeus japonicus*. Book of abstracts in the World Aquaculture society, Taipei, Taiwan.
2. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., 2017. Interactive effects of dietary astaxanthin and vitamin E on the performances of kuruma shrimp, *Marsupenaeus japonicus*. Book of abstracts in the 11th Symposium of World's Chinese Scientists on Nutrition and Feeding of Finfish and Shellfish, Huzhou, China.
3. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., 2017. Optimum supplementation of astaxanthin for kuruma shrimp, *Marsupenaeus japonicus*. Book of abstracts in the World Aquaculture society, Kuala Lumpur, Malaysia.
4. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., 2016. Optimum supplementation of Astaxanthin for larval and post-larval kuruma shrimp, *Marsupenaeus japonicus*. Book of abstracts in the Tenth Symposium of World's Chinese on Crustacean Aquaculture, Shanghai, China.
5. **Wang, W.L**., Ishikawa, M., Koshio, S., Yokoyama, S., 2016. Optimum supplementation of Astaxanthin for juvenile kuruma shrimp, *Marsupenaeus japonicus*. Book of abstracts in the Carcinological Society of Japan, Kagoshima, Japan.

List of Abbreviations

ANOVA:	Analysis of Variance
Ax:	Astaxanthin
BW:	Body Weight
BWG:	Body Weight Gain
CMI:	Cumulative Mortality Index
DS:	Developmental Stage
FCR:	Feed Conversion Ratio
FI:	Feed Intake
HPLC:	High Performance Liquid Chromatograhpy
3-HUFA:	n-3 Highly Unsaturated Fatty Acids
LT_{50}:	The time required 50% mortality of test shrimp by stressors
MBD:	Microbound Diet
ProPO:	Prophenoloxidase Activity
SGR:	Specific Growth Rate
SR:	Survival Rate
THC:	Total Haemocyte Count
TL:	Total Length
VC:	Viable Cell
SEM:	Standard Error of Mean

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I would like to express my gratitude to all those who helped me during the doctoral course. My deepest gratitude goes first and foremost to Dr. Manabu Ishikawa, Professor, for his acceptance to me as his student, also his guidance, advice, interest, belief, understanding, encouragement throughout my doctoral course. I also would like to thank Dr. Sunsuke Koshio, Professor and Dr. Saichiro Yokoyama, Assistant Professor for their encouragement, suggestions and training on analytical procedures and operations.

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Chapter 1: General Introduction

General Introduction of Kuruma Shrimp Culture and Market

Shrimp is by far the most important commodity by value in the international fish trade. Yearly exports of shrimp and shrimp products now exceed more than US $ 10 billion (2016) and represent almost 20% of world total exports of fish and fishery products. Shrimp ranks regularly among consumers' favorite seafood and is consumed in all parts of the world, with the largest markets being the United States, Japan, and the European Union.

It is well known that shrimp today comes from both capture fisheries and aquaculture. Output from shrimp aquaculture has more than doubled over the last few decades, and a growing share of internationally traded shrimp is now coming from aquaculture. Shrimp aquaculture has an important impact on supply and demand patterns in all markets. Kuruma shrimp (*Marsupenaeus japonicus*) as the warm-water shrimp is considered one of the most economically important members of the penaeidae family. It is also fished in various parts of its natural range, but its greatest importance is in aquaculture. Kuruma shrimp culture has been practiced in Japan for many decades. Since, aquaculture is the only viable way of bridging the gap between demand and supply of this high-value seafood (Bulbul et al., 2014). Since 2003, more than 39,000 tonnes (Fig. I) has been produced in shrimp farms annually, and the quantities of the annual catch declined year by year since 1990 (Fig. II).

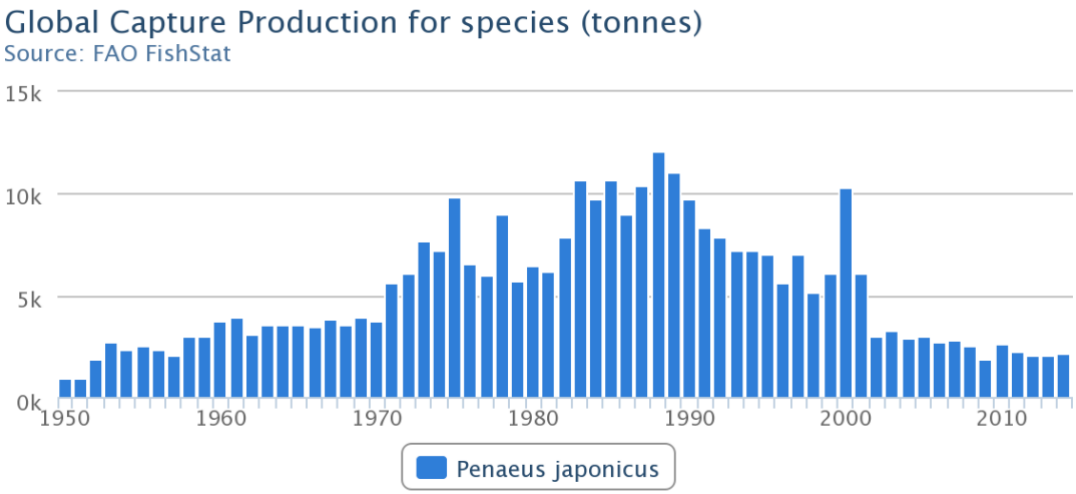

Fig. I. Date of global capture production for kuruma shrimp.

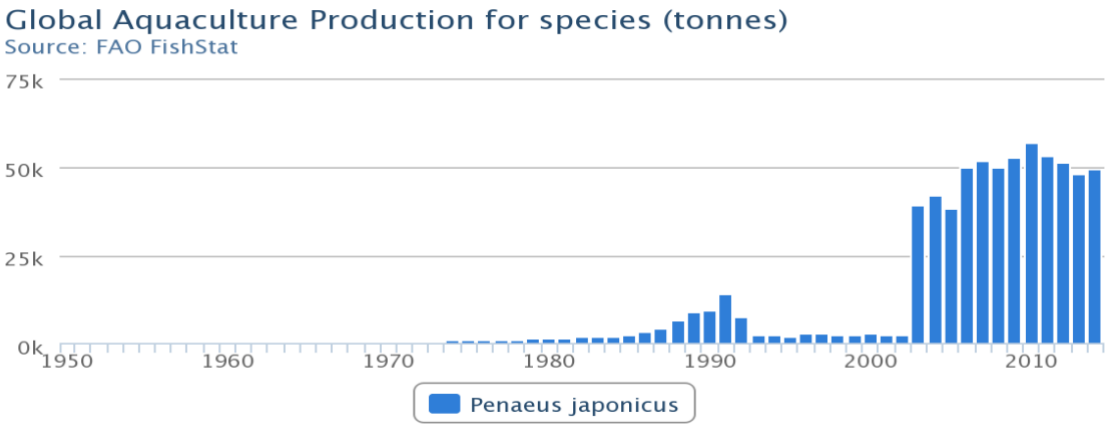

Fig. II. Date of global aquaculture production for kuruma shrimp.

Despite growing output, shrimp and shrimp products are still generally considered high-value species with high appeal among market consumers and strong demand also from the catering of restaurant sector. In fact, with increasing production and trade, availability has also increased and restaurants all over the world are today able to offer shrimp as part of their regular menu. However, with shrimp prices often showing large fluctuations, this means that changes in the shrimp price soon translate into price changes on the menu or on the supermarket shelf. Maintenance of natural pigmentation is of utmost importance from a commercial perspective, being directly associated with the perception and subjective interpretation of consumer as an important quality criterion prior to actual consumption which consequently commands a better demand and product market price. For this reason, color exerts a strong decisive role when evaluating and determining the quality of the product at the point of sale. The progressive expansion of aquaculture industry has established an insatiable demand for the carotenoid pigment.

Structural and Biological Aspects of Pigments

The coloration change of the crustaceans can be due to physiological or morphological mechanism (Wade et al., 2012). The influence of carotenoid availability in the diet or from culture environment, photoperiod, light intensity, and temperature are considered as the

physiological mechanisms (Rao, 1985). Such color changes induced by physiological mechanisms are often rapid, reversible, and rhythmic. These changes are associated with hormonally controlled expansion and contraction of pigment structures, known as chromatophores, contained within the hypodermal layer, the pigmented layer in between the exoskeleton and abdominal muscle. In contrast to the physiological, the morphological mechanisms are considered to be slower and more permanent which involve quantitative modifications of exoskeletal and hypodermal layer pigment concentration or composition (Wade et al., 2012). The traditional view is that physiological and morphological mechanisms of color change are independent of each other. However, the relationship between them has not been investigated.

Within the exoskeleton and hypodermal tissue, Ax is often bound within a multimeric protein complex called crustacyanin (CRAN). This interaction modifies the naturally red carotenoid, producing the diverse array of colours seen in the exoskeleton of crustaceans. During cooking, this interaction is disrupted, releasing the distinct red coloration of cooked seafood.

Astaxanthin as Feed Supplement in Aquatic Animals

Ax (3,3′ –dihydroxy-β, β′-carotene-4,4′-dione) is an oxidized form of β-carotene being widely distributed in nature and largely discovered in the marine environment (Lim et al., 2017). This carotenoid pigment is abundant in the flesh of salmonids, carapace of many crustaceans (e.g. shrimp, crabs, lobsters and crayfish) and also in other marine organisms such as microbes (Asker et al 2012) and microalgae (Stewart et al., 2008). Aquatic animal generally exhibit poor ability to biochemically synthesize Ax de novo and thus require diets containing Ax to acquire the appropriate coloration (Kim et al., 2006). Naturally, the carotenoid pigment Ax is primarily biosynthesized in microalgae within the food chain at the primary production level. Microalgae are then consumed by crustaceans, zooplankton or insects that amass the Ax and in turn transferred to the higher trophic levels when ingested by fish and other aquatic animals.

High-density shrimp farming systems are widely used by farmers now. However, kuruma shrimp cultured under intensive culture conditions with artificial diets are subjected to lose its natural colors (Chien and Jeng, 1992). Maintenance of the natural skin pigmentation is considered very important from a commercial point of view which directly associate with the product market price and the choice of consumers (Shahidi and Brown, 1988). Therefore, it is necessary to add pigments to diets to enhance the color of shrimp and improve their commercial value.

Niu et al. (2012) illustrated that dietary supplementation of carotenoids can improve or correct the color of penaeids for a better market price. The inclusion of pure carotenoids (β-carotene, echinenone, canthaxanthin and zeaxanthin) and crude carotenoids (oleoresin paprika, crayfish waste extract, corn gluten, alfalfa, and spirulina) in diets improves pigmentation in crustaceans through various biosynthetic processes, as described by Tanaka et al. (1976). However, Okada et al. (1994) indicated that Ax is the predominant pigment (86-98% of total carotenoids) in penaeids and other crustaceans. Moreover, main pigment usually being Ax in the free or esterified form in crustaceans (Niu et al., 2012). Ax is the most effective pigment (Chien and Jeng, 1992).

Ax plays a fundamental role not only in the cultivation but also in the breeding of diverse kinds of aquaculture species. To data, there exist many lines of evidence to suggest that Ax confers a significant impact on reproductive performance, egg production and egg quality of aquatic animals (Tizkar et al. 2013; Palma et al., 2016). It is generally accepted that Ax plays a vital role in the intracellular intermediary metabolism of aquatic animals (Kiron, 2012). This would consequently affect the physiological functions and further enhance the nutrient utilization or assimilation of aquatic animals ultimately resulting in intensifying growth performance (Amar et al. 2001). High-density aquaculture operations frequently subject animals to various physical stressors which involve grading, transport, handling, vaccination, crowding and confinement or any other forms of physical disturbance that could be extremely stressful and immune-depressive. Presented data constantly imply the positive impacts of dietary Ax on antioxidant capacity, stress alleviation, immune response regulation and disease resistance of aquatic animals. The implicated biological functions of Ax can be largely ascribed to its supreme antioxidant properties.

Ax is a hydrophobic xanthophyll which exhibits poor solubility in the aqueous environment of the gastrointestinal tract. Gastrointestinal absorption and metabolism of Ax are known to be strongly influenced by the presence of certain dietary factors which include cholesterol, fatty acids and vitamin E. Ax is known to be transported alongside these molecules through the intestine and blood. Most of the available reports describing the metabolism of Ax in aquatic animals were assessed on salmonids.

Carophyll Pink

Carophyll Pink is a formulated product that provides the feed manufacturer with a stable and reliable source of nature identical Ax. It is produced intensively with a carefully documented low environmental impact.

In Carophyll Pink, the sensitive Ax molecules are stabilized with antioxidants and embedded in a carbohydrate and gelatin matrix. This stable product is coated with starch to improve handling. During the manufacture of fish feed Ax is liberated from Carophyll Pink. This is a necessary step to increase the bioavailability of the previously encapsulated Ax. Stability is linked to bioavailability there is a trade off between these two factors.

Bioavailability is defined as retention in fish flesh. Many scientific studies have shown that Carophyll Pink added pre-extrusion ensures optimum Ax bioavailability. Furthermore, these studies have also demonstrated that the nature identical free form, of Ax derived from Carophyll Pink results in the highest deposition efficiency in cultured fish and shrimp.

Color Space

A color space is a specific organization of colors. In combination with physical device profiling, it allows for reproducible representations of color, in both analog and digital representations. When defining a color space, the usual reference standard is the CIELAB or CIEXYZ color spaces, which were specifically designed to encompass all colors the average

human can see. Lab color space is a color-opponent space with dimension ***L**** for lightness and ***a**** and ***b**** for the color-opponent dimensions, based on nonlinearly compressed coordinates.

Objectives

Kuruma shrimp (*Marsupenaeus japonicus*) has become one of the most widely cultured shrimp species because of its high quality (Bulbul et al., 2013). With the greater expansion of aquaculture activities during last two decades, some differences on quality were discovered compared with the natural shrimp. One of the problems is color of kuruma shrimp flesh. Color is one of the major factors that determine the price of live kuruma shrimp (Yamada et al., 1989). The pink of cooked shrimp is one of the most attractive attributes and important quality criterion for most consumers (Lucien-Brun and Vidal 2006). Thus, methods that improve the red color development are of significant industrial importance. There is no information on the optimal supplementation level of dietary Ax for different stages of kuruma shrimp. Furthermore, Ax is reported that exhibits poor solubility in the aqueous environment of the gastrointestinal tract. Certain dietary factors such as vitamin E, and cholesterol are known influence absorption of Ax in aquatic animals. Information on Ax mechanism is mostly derived from a limited number of studies almost on salmonids. Therefore, the objectives of this study were:

(1) to evaluate the effects of synthetic Ax (Carophyll Pink) on larval and post-larval kuruma shrimp and to ascertain an optimum supplemental level on them.

(2) to evaluate the effects of synthetic Ax (Carophyll Pink) on juvenile kuruma shrimp and to ascertain an optimum supplemental level on it.

(3) to evaluate the interactive effects of dietary synthetic Ax (Carophyll Pink) and vitamin E on growth performances, stress resistance, immune response, and pigmentation on juvenile kuruma shrimp.

(4) to evaluate the interactive effects of dietary synthetic Ax (Carophyll Pink) and cholesterol on growth performances, stress resistance, immune response, and pigmentation on juvenile kuruma shrimp.

Chapter 2: General Materials and Methods

Preparation of the Test Diets

Micro-bound diet (MBD) was prepared for larval shrimp according to the method described by Teshima et al., (1983). All dry ingredients were crushed in the mortar before weighting and mixing when particles sizes were bigger than 53um diameters. Lipid ingredients were then added and thoroughly mixed well.

Then dissolved carrageenan into water at 85˚C. Well mixed dry and lipid ingredients were added into it and stirred well until the mixture became a pudding-like consistency and adjusted pH (7.0-7.5) with 1N sodium hydroxide. The dough was cooled to room temperature before putting into the refrigerator. Freeze drying and powdered and sieved into the desire particles sizes were then carried on. Then the diets were kept in -20˚C refrigerator until use.

Pellet diet was prepared for juvenile shrimp. The diets were prepared by thoroughly mixing all the dry ingredients in a blender for 15min. All the lipid sources were premixed together with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), then added to dry ingredients and mixed for another 15 min. Finally, water (35-40% of the dry ingredients) was added to the premixed ingredients and mixed for another 15min. The pH of mixture was adjusted to 7.0-7.5 with 1N sodium hydroxide. The mixture was then molded into an appropriate diameter (1.2 to 2.2mm) to prepare pellets using a single-screwed mincer (ROYAL Inc., Tokyo, Japan). Pellets were then dried in a dry-air mechanical convection oven (DK400, Yamato Scientific, Tokyo, Japan) at 60˚C for 60 min. The dried pellets were steamed at 100˚C for 1 min in a cylindrical steamer to improve water stability and stored at -20˚C until use.

Sample Collection

At the end of the feeding trial, all treatment groups were fasted for 24 h prior to final sampling. The total numbers of survivors and individual body weight from each tank were measured using following formulae:

Body weight gain (BWG, %) = [(final weight-initial weight)/initial weight] × 100

Specific growth rate (SGR, % day^{-1}) = [(Ln final weight-Ln initial weight)/duration] × 100

Feeding intake (FI, g/shrimp/56 days) = total feed intake/shrimp number/duration

Feed conversion ratio (FCR) = dry weight of feed consumed (g)/live weight gain (g)

Survival (%) = (final number of shrimp/initial number of shrimp) × 100

Hepatopancreas index (HSI, %) = hepatopancreas weight/body weight × 100

Three juveniles from each tank were obtained for immunological analysis. A quantity of 200 µL haemolymph was collected from the ventral sinus of shrimp with 800 µL of sterile anticoagulant (NaCl 29.22 g L^{-1}, EGTA 3.8 g L^{-1}, Hepes 2.38 g L^{-1}, and *L*-cysteine 5 g L^{-1} of stock solution) using a 1 mL syringe (Terumo Corp., Tokyo, Japan). The mixture was immediately placed into a vial, chilled on ice and transported to the laboratory for further analysis.

Analytical Procedure

Proximate Composition Analysis

The ingredients, diets and shrimp whole body were analyzed for moisture, crude protein, lipid and ash in triplicate using standard methods (AOAC 1990).

Moisture content: whole body samples were dried until the weight became constant by using freeze dryer (Eyela freeze dryer FD-1, Tokyo Rikakikai Co. Ltd., Japan). Diet samples were dried to constant weight by dry air mechanical convection oven at 105˚C. Losses in weight represented moisture content.

Moisture (%)=(W1-W2)/W1x100

W1=Sample weight before drying

W2=Sample weight after drying

Crude protein: Kjeldahl method was contributed to analyze the crude protein. Approximately 0.2g dried samples and 2g catalyze (K_2SO_4: $CuSO_4$=9:1) were digested in 10ml concentrated H_2SO_4 and 10ml 30% H_20_2 in Kjeldahl flask by heating for 90min at 450˚C with dissolving apparatus (Kjeldahl system 1007, Tecator, Sweden). 70ml deionized water were added into the samples, the mixture was steam-distilled in 4ml 40% NaOH, using the distilling apparatus (Kjeldahl system 1002, Tecator, Sweden). Approximately 150ml distilled into H_3BO_3 solution, with bromcresol green and methyl red indicator in ethanol, was titrated with 0.1N H_2SO_4 solution to a neutral pH value. Percentage of N and crude protein were calculated as follows:

N(%)=[14.008 x (A-B) x F x 0.1]/(S x 10)

Crude protein (%)=6.25 x N (%)

A= The volume of H_2SO_4 solution titrated for sample

B= The volume of H_2SO_4 solution titrated for blank

F= Factor of 0.1N H_2SO_4 solution

S= Sample weight (g)

Crude lipid: Soxhlet method was contributed to analyze the crude lipid. 5g samples were weighed and placed in an extraction thimble and covered with cotton. 150ml of diethyl ether was used for extracting lipid from the sample using Soxhlet extraction apparatus for 6 hours. The flask with the extracted lipid was then dried in rotary vacuum evaporator (Eyela SB 1100, CCA 1111) at 34˚C. Flasks were then dried in oven at 90-100˚C for constant weight. Crude lipid was then calculated as follows:

Crude lipid=[after flask weight-before flask weight]/dry sample weight x 100

Ash content: dried samples of weight 1g were placed in crucibles with covers and laid in muffle furnace (Hayashi Denko Co., Ltd. Japan) at 550˚C until the weight became constant. The samples were weighed after cooling in desiccators. And ash content was calculated as follows:

$$\text{Ash (\%)} = (\text{weight of ash}/\text{weight of sample}) \times 100$$

Stress Resistance Test

Fresh water-osmotic stress test was conducted after feeding trial. Juvenile shrimp was challenged with low salinity condition to investigate the tolerance against stressors and evaluate the efficient of test diets. After the feeding trial, five shrimp from each rearing tank were randomly selected and transferred in the 20L tank with 10L seawater. Then letting fresh water to flow in until 0 ppt salinity was reached in 30 minutes. Once 0ppt salinity was obtained, survival was checked followed every hour. Cumulative mortality (%) was transformed into common $\log_{10}$ (% survival) to fit a regression line and the mean LT_{50} was calculated by the regression equation (Borgmann et al., 1978; Yokoyama et al., 2005).

Formalin stress was conducted to assess the quality of tried shrimp larvae. Test solution was prepared by mixing filter seawater with 10% formaldehyde buffer solution (Nacalai Tesque, Tokyo, Japan) at 150ppm concentration for larval stages (Samocha et al, 1998). Ten test shrimps from each experimental tank were randomly collected and placed into a beaker, which was placed inside the water bath and filled with 100ml test solution without supplying aeration. The time when shrimp died after exposure to formalin solution was recorded to calculate the value of LT_{50}.

LT_{50} is defined that the time required 50% mortality of test shrimp by stressors. The calculation was done as follows: time to death (min) was converted to log10 values. When the shrimp were exposed to the solution at first, they were still alive. Therefore, the survival was assumed to be 100%, which is converted to log value ($\log_{10}100=2$). The calculation was conducted base on the data as every minute. These values of log survival were plotted against the time of the death to determine the duration of 50% mortality of shrimp in each treatment.

Each value obtained from the equation above was compared statistically. The higher value indicates the greater tolerance against the stressors. The equation is following:

$Y = a X + b.$

$Y = \log_{10}(\%survival)$

X= time to individual death of shrimp

LT_{50} (X) was obtained when Y=1.7

Total Haemocyte Count, Viable Cells and Phagocytic Activity Assay

The total haemocyte count (THC), viable cell (VC) and the rate of phagocyte activity (PA) were assayed as indicators for Immunological assay. The method described by Itami et al (1994 and 1998) was applied with slight modification. 200*u*l haemolymph was collected from the ventral sinus with 800*u*l of sterile anticoagulant (NaCl 29.22g/l, EGTA 3.8g/l, Hepes 2.38g/l, L-cysteine 5g/l) by using 1ml syringe, then centrifuged at 1000rpm at 5˚C for 10min by centrifuge (MX160, Tomy, Tokyo, Japan). The supernatant was discard and pellet was washed with 1ml shrimp saline solution (NaCl228.4g/l, $MgCl.6H_2O$ 1g/l, $MgSO_4.7H_2O$ 2g/l, $CaCl_2.2H_2O$ 2.25g/l, KCl 0.7g/l, Glucose 1g/l, Hepes 2.38g/l) by centrifugation three times. The sediment was re-suspended in shrimp saline solution and divided into two equal sub-samples, one for measure of total haemocyte count (THC), viable cell (VC) and the other to measure phagocyte activity.

For total haemocyte count, the diluted haemocyte was mixed with equal volume of trypan blue and counted VC using haemocytometer (Burker-Turk, Minato Medical Corporation, Tokyo, Japan).

Viable Cell (%)=Non-stained cells/total haemocytes x100

For phagocyte activity, 200*u*l haemocytes at concentration $1x10^6$ cells/ml were spread on cover slip (size 22 x 24 x 0.4mm) in 6 well-plates with cover and mixed with heat-killed yeast ($5x10^8$cells/ml). After incubation at 25˚C for 2 hours, the samples were fixed by Fixative (4%

Paraformaldehyde in 0.2M Sodium Cacodylate buffer with 10% sucrose). Then they were stained by Wright solution (Wako Pure Chemical Industries, Ltd., Japan) and mounted with Eukitt (Entellan, Merck, Germany) for yeast method. On the micro-glass cover and observed under microscope. The number of phagocytosis cell was counted from 200 cells and the rate of phagocyte activity (PA) was calculated by equation:

$$PA\ (\%) = \text{Phagocytosis cells/total haemocytes} \times 100$$

Prophenoloxidase Activity Assay

The prophenoloxidase system is considered a constituent of the immune system and is probably responsible for the nonself recognition process of the defense mechanism in crustaceans and insects (Ratcliffe et al., 1985). Prophenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-DOPA with the method described by Hernandez-Lopez (1996) with slight modifications as follows: 200*u*l haemolymph was collected from the ventral sinus with 800*u*l of sterile anticoagulant by using 1ml syringe, then centrifuged at 6500rpm (2700xg) at 4˚C for 20min. Discarded the supernatant fluid and the pellet was rinsed by shrimp saline for three times, and re-suspended gently in 1ml cacodylate-citrate buffer (Sodium cacodylate 0.01M, Sodium chloride 0.45M, Trisodium citrate 0.01M). After centrifuged at 10000rpm (6500xg) at 4˚C for 10min again, the supernatant was collected as HLS (Hemocyte lysate supernatant). Aliquot 100*ul* of HLS was incubated for 10min at 25˚C with 50*ul* of trypsin (1mg/ml Cacodylate buffer). 50*u*l of L-DOPA (4mg/ml Cacodylate buffer) was then added for 10min at 20˚C followed by 800*ul* of cacodylate buffer 5 min later. The optical density at 490nm was mensured every 5 min using a DR 4000-HATCH spectrophotometer (HATCH Company, USA). The control solution was used for the background phenoloxidase in all test conditions and consisted of 100*ul* cell suspension, 50*ul* L-DOPA and 50*ul* of cacodylate buffer (to replace the trypsin).

Analysis of Digestive Enzymes

The hepatopancreas samples were collected in 1 h after the final sampling to guarantee maximum activity of digestive enzymes. The hepatopancreas of shrimp were dissected out, weighted and homogenized with shrimp saline shrimp saline solution (NaCl 28.4 g L^{-1}, $MgCl{\cdot}6H_2O$ 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 2 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 2.25 g L^{-1}, KCl 0.7 g L^{-1}, Glucose 1 g L^{-1}, Hepes 2.38 g L^{-1}) in ice cooled condition. The homogenate was centrifuged at 6000 rpm for 20 min at 4℃. After centrifugation, collecting the supernatant fluid. The samples were stored at -80℃ until analysis.

Protease activity was analyzed with the method of Sigma's Non-specific Protease Activity Assay (Cupp-Enyard, 2008) with slight modifications. The enzyme assays were conducted with using casein as the substrate and reacting with Folin-phenol reagent. The 0.5 mL of diluted enzymatic extract for each sample were incubated at 37℃ for 10 min with adding 5 mL of 0.65% casein. Then stopping the reaction by adding 5 mL 110 mM trichloroacetic acid (TCA) for 30 min. Filter the mixture using a 0.45 μm syringe filter, and then 5 mL 500 mM sodium carbonate solution and 1 mL of 0.5 mM Folin's reagent were added and incubated at 37℃ for another 30 min. After incubating, using the 0.45 μm syringe filter to filter again for the better results measured by the spectrophotometer (Hitachi High-Technologies Corportaion, Japan) at A_{660} versus blank.

Amylase activity was analyzed with the method of Shiu et al. (2015) with slight modifications. Incubate the tubes added with 0.5 mL enzyme solution for 3-4 min to achieve temperature equilibration. At timed intervals, add 0.5 ml starch solution and incubate exactly 3 min at 25℃. At timed intervals add 1 mL dinitrosalicylic acid color reagent to each tube and incubate another 5 min in a boiling water bath. Then cool to room temperature and add 10 mL distilled water. Mix well and read by spectrophotometer at A_{540} versus blank.

Lipase activity was analyzed with the method of Mustafa et al. (2016) with slight modifications. The enzyme assays were conducted by using olive oil emulsion as the substrate, and produced fatty acid was extracted by isooctane, then the extracted fatty acid was determined by copper soap colorimetry measurement. The olive oil emulsion was prepared by mixing 50 mL of olive oil and 150 mL 4% polyvinyl alcohol solution in a homogenizer. The reaction mixture containing 2 mL of olive oil emulsion, 2.5 mL of 20 mM phosphate citrate buffer (pH 7.5) and 0.5 mL enzyme solution was incubated at 40℃ for 15 min. The reaction

was then immediately stopped after incubation, by adding 1 mL 6 M HCl and 6 mL 95% ethanol. After mixing well, collecting 1 mL of the supernatant fluid and adding 4 mL of isooctane and 1 mL 5% cupric acetate pyridine reagent (pH 6.1) for reading by the spectrophotometer at A_{714} versus blank.

Astaxanthin Analysis

The shrimp samples were freeze dried and then crushed to form a powder. The samples were then placed into a 50 mL polypropylene centrifuge tube for Ax analysis following the method of Masako et al. (2010), with slight modifications. The Ax was then extracted with chloroform by ultrasonic mixing for 10 min and was centrifuged at 4°C and at 11800 rpm for 15 min. The extracted Ax was transferred to flasks and dried in rotary vacuum evaporator (Eyela SB 1100, CCA 1111). The sample extract was then dissolved in 10 mL acetone solution. A volume of 0.05 M Tris-HCl buffer (pH 7.0) 6 mL and cholesterol esterase solution (60 unit mL^{-1}) 600 µL was added to the extract, and the enzyme reaction allowed to complete for 120 min at 37°C. After the enzyme reaction, the solution was transferred to a 250 mL separatory funnel, partitioned with 10 mL petroleum ether. The solution was washed three times with 0.01 g $NaSO_4$ to remove residual acetone. Then, using the rotary vacuum evaporator again, the solvent was mobilized and filtered through a 0.2 µm Millipore filter and stored in brown vials. Ax was analyzed by high-performance liquid chromatography (HPLC), using a shim-pack VP-ODS column (150 mmL. × 4.6 mmL.D.) and a SPD-M20A detector at 475 nm. The operational conditions were: mobile phase, 0.05% trifluoracetic Acid/methanol = 3/97 (v/v) and solvent flow rate, 1.2 mL min^{-1}; injection volume, 10 µL. This system was controlled by a chromatographic data system (Scientific Information Services Corporation), which also integrated the areas under the peaks. The standard was prepared using 8% Ax contained in Carophyll Pink®. The standard was prepared for five levels (0.05-20 mg L^{-1}) using 8% Ax contained in Carophyll Pink®.

Fatty Acid and Cholesterol Analysis

Fatty acid analysis was analyzed using a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector using an Omegawax 320 column (Supelco, Inc., Japan). Fatty acid methyl esters were prepared using the shrimp body according to the method of Bligh and Dyer (1959) and then further separated into neutral lipid (NL) and polar lipid (PL) fractions by Sep-pak silica cartridge (Waters Corporation, Milford, Massachuseetes, USA) as described by Juaneda and Rocquelin. (1985). Both fractions were analyzed to quantify the content of CHO from lipid classes analyzed by MK-6s Iatroscan equipped with a flame ionization (Iatron Lab., Japan) (Thongrod and Boonyaratpalin, 1988). Peaks were integrated with a chromatopac C-R8A (Shimadzu, Japan). Methylating rest of the NL and PL by adding calculated amount of C23:0, respectively. Keeping samples into the heat reactor for 1 h with 1 mL boron trifluoride and 0.5 mL dichloromethane. After that, mixing well with 0.5 mL NaCl and 1 mL hexane, and taking the upper layer for further fatty acid analysis.

Statistical Analysis

The statistical analyses were performed by using an analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). All data were presented as mean values standard ± error of mean (S.E.M., n=3), Data from each group were compared using Tukey Kramer test. Differences between treatments were considered significantly when $P < 0.05$. Two-way ANOVA was employed to test the effects of dietary two additives levels, as well as their interactions. If no interaction, One-way ANOVA was attributed to each individual factor to evaluate the inclusion level of each factor (Dawood et al., 2015b; Michael et al., 2006).

Chapter 3: Effects of Dietary Astaxanthin Supplementation in Kuruma Shrimp

Study 1: **Effects of Dietary Astaxanthin Supplementation on Survival, Growth and Stress Resistance in Larval and Post-larval Kuruma Shrimp, *Marsupenaeus japonicus***

Abstract

Two trials were conducted to evaluate the effects of chemically synthesized astaxanthin (Ax) on growth performance, survival, and stress resistance in larval and post-larval kuruma

shrimp (*Marsupenaeus japonicus)*. Six dietary levels of Ax (0, 50, 100, 200, 400, and 800 mg kg^{-1} diet) were added to a baseline diet. As a first study, an 8-day feeding trial was conducted on larval kuruma shrimp, with results showing that larvae fed diets supplemented with different Ax levels exhibited better performance during developmental and metamorphosis to post-larvae. Broken-line regression analysis indicated that the optimal levels for growth and stress resistance of larvae were 168.9 mg kg^{-1} and 82.1 mg kg^{-1} diet, respectively. Secondly, a 30-day feeding trial was conducted on post-larval shrimp. Supplementation with 100 and 200 mg kg^{-1} Ax yielded significantly higher final body weight, body weight gain, and specific growth rate than that in a control group. A cumulative mortality index for osmotic stress revealed significantly better performance in a group fed 200 mg kg^{-1} supplementation than in controls. Broken-line regression analysis indicated that the optimal levels for growth and stress resistance of post-larvae were 108.7 mg kg^{-1} and 178.1 mg kg^{-1} diet, respectively. Based on these results, I suggest that carefully dosed Ax supplementation is a beneficial nutritional strategy for the early developmental stages of kuruma shrimp.

Keywords: astaxanthin, kuruma shrimp, larval, optimal level, post-larval

Introduction

Kuruma shrimp (*Marsupenaeus japonicus)* are the most important cultured crustacean species in Japan owing to their high market value and demand (Chien & Jeng 1992). However,

the wild catches of kuruma shrimp decreased to 700 tons in 2008 (FAO 2010). Recently, the use of intensive aquaculture systems has increased and is emerging as a practical and promising tool to meet the high demand.

Astaxanthin (Ax) is a pigment well known to produce red coloring in shrimp. Previous studies have investigated Ax supplementation in juvenile farmed shrimp, which can yield body coloring approximating that of wild shrimp which attracts a higher price on the international market (Kalinowski et al. 2005; Niu et al. 2009). In addition, Ax has also been reported to have several positive biological effects on shrimp by enhancing maturation, antioxidant activity, anti-inflammatory activity, immune response, and stress resistance (Chien & Jeng 1992; Merchie et al. 1998; Lorenz & Cysewski 2000; Pan et al. 2001; Chien et al. 2003; Guerin et al. 2003). Moreover, dietary administration of Ax can enhance the growth and survival of post-larval black tiger shrimp, *Penaeus monodon* (Thongrod et al. 1995; Pan et al. 2001). Ax may also be an essential growth factor in the early development of *Penaeus esculentus* (Dall et al. 1995). Furthermore, Ax also improves the stress tolerance of post-larval white shrimp, *Litopenaeus vannamei* (Niu et al. 2009) and the carotenoid pattern of *Penaeus japonicus* during the larval stage (Petit et al. 1998). However, there are no data available concerning about the effects of dietary Ax on the larval stages of kuruma shrimp, or its optimum supplementation for larval and post-larval kuruma shrimp. Therefore, we conducted two individual trials on kuruma shrimp to determine the effects of chemically synthesized Ax on the growth performance, survival, and stress resistance, and to evaluate the optimum supplementation levels of Ax.

Materials and Methods

Astaxanthin Preparation

A chemically synthesized pigment (Carophyll Pink®, DSM Nutrition, Japan) containing 8 % Ax was used for the experiments. The relatively high proportion (about 94 %) of Ax in total carotenoids in this source indicates that its pigmentation efficiency is mainly due to Ax.

Shrimp and Test Diet

Fifteen wild catches of mature female kuruma shrimp were obtained from Matsumoto Suisan, Miyazaki, Japan, and transported to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University. Shrimp were maintained at ambient water temperature (27 ± 0.5 ℃) in a 500 L black polyethylene circular tank containing approximately 400 L filtered seawater until spawning occurred. After removal of the spawner, hatched nauplii were raised to 1st zoea stage for larval experimentation. For post-larval experiments, animals at the 20th post-larval stage (7.9 ± 0.2 mg initial wet weight) were used.

The basal composition of test diets is shown in Table 1-1. Casein with added *L*-arginine (Teshima et al. 1986) was used instead of fishmeal as the major protein source in order to ensure that the supplemented Ax was an isolated variable. Squid liver oil, cholesterol and soybean lecithin were used as the major lipid sources. Mineral and vitamin mixtures were added according to Moe et al. (2004) and Alam et al. (2004), though without vitamin A so as to ensure Ax availability. The diet was micro-bound using *k*-Carrageenan as a binder according to Teshima et al. (1983) and Koshio et al. (1989). The micro-bound diets (MBD) were sieved for desired particle sizes, as follows: 53-125 μm diet for zoea stages; 125-250 μm for the 1st and 2nd mysis stage; 250 μm for the 3rd mysis and 1st post-larval stages; 500-700 μm and 700-1000 μm for post-larval experiment. Sieved MBD were stored at -20℃ before and during the experiments. Ax was supplemented at six levels, including 0 (control), 50, 100, 200, 400, and 800 mg kg^{-1} diet. Cellulose powder was used to adjust to 100 % of the total proportion.

Larval Feeding Trial

The feeding trial was conducted in the Kamoike Marine Production Laboratory. A 300 L rectangular tank was filled with 50 L flowing seawater. Eighteen 1 L beakers were set up on the bottom of the rectangular tank, with each dietary treatment represented in three replicates. Beakers were filled with filtered seawater and gently aerated via capillary pipes, with temperature monitored daily. One hundred 1st stage zoea larvae were randomly distributed in each beaker. Beakers were cleaned and water completely replaced every morning at 0600. Test

diets were fed three times a day, at 0800, 1200, and 1700. Feeding amounts were 0.08 mg/larvae/day for zoea stages, 0.16 mg/ larvae/ day for the 1st and 2nd mysis stage, and 0.32 mg/larvae/day for the 3rd mysis stage and 1st post-larval stage, following the methods described by Teshima et al. (1983).

Larval growth was evaluated daily based on 10 random samples, as per Hudinaga (1942). Survival rates were also determined daily by counting all the surviving larvae. At the end of feeding trial, all surviving larvae were counted and assessed microscopically to determine the developmental stage.

Larval performance was evaluated based on percentage survival (SR), total body length (TL), developmental stage (DS), percentage of larvae metamorphosed to the1st post-larval stage (PL), and tolerance to formalin solution exposure stress (LT_{50}). SR was calculated based on the number of surviving larvae and PL was calculated based on the ratio of surviving larvae that reached the 1st post-larval stage. For TL, 10 larvae from each beaker were randomly collected and measured individually using a digital caliper. DS was determined based on the following equation (Koshio et al. 1989):

$$DS = (n_1+2n_2+3n_3)/(n_1+n_2+n_3)$$

where n_1 = number of mysis 2nd, n_2 = number of mysis 3rd, n_3 = number of post-larvae.

Post-larval Feeding Trial

Kuruma shrimp (20th post-larval stage; 7.9 ± 0.2 mg initial wet weight) were randomly selected from stock tanks and transferred to prepared 40 L transparent rectangular tanks. Fifty shrimp per tank were distributed into each treatment tank, with three replicates per treatment. Seawater was continuously supplied at a flow rate of 130 ml min^{-1} and gently aerated via capillary pipes and air stones. Water temperature was kept at 26 ± 2 ℃ and kept in natural light. Post-larvae were fed with MBD twice a day during the trial, using 500-700 μm food particle size for the first 15 days and 700-1000 μm particle size for the last 15 days, over 30 days in total. Mortality and water conditions were observed every morning and evening throughout the trial. Uneaten feed and feces were removed in the morning prior to feeding.

Every 10 days, all post-larvae in each tank were counted to monitor their survival and bulk-weighted to adjust the feeding rate. The efficacy of Ax was evaluated based on survival (SR), specific growth rate (SGR), body weight gain (BWG), tolerance against formalin solution exposure stress (LT_{50}) and fresh water-osmotic stress (CMI).

Stress Resistance Test

Formalin exposure and freshwater osmotic stress tests were conducted after feeding trials, where the shrimp were challenged with a formalin solution or low salinity to evaluate tolerance to stressors and the efficiency of the test diets. Formalin solution exposure stress tests were conducted for both larval and post-larval shrimp. Freshwater osmotic stress testing was conducted only for post-larval shrimp, since the osmoregulatory capabilities of larval shrimp are limited in comparison to juvenile and adult penaeid shrimp (Castille & Lawrence 1981) and the ability to osmoregulate at low salinities develops after metamorphosis to post-larvae (Samocha et al. 1998). The formalin stress test is a rapid, inexpensive, and simple procedure that is used as a shrimp quality assessment measure (Samocha et al. 1998). The response to osmotic or chemical stress is dependent upon the age and nutritional status of the post-larvae (Tackaert et al. 1989).

For the freshwater osmotic tress test, ten test shrimp post-larvae from each experimental tank were put in 1 L beakers with 800 ml dechlorinated tap water. Mortality was monitored every 5 min. Survival was expressed as the cumulative mortality index (CMI) and calculated as the average value of cumulative survival obtained from each replicate diet in relation to the experimental time (Tung et al. 2010). A lower value indicates greater tolerance to the stressors. CMI was calculated according to the following equation:

$$CMI = M_1 + M_2 + \ldots + M_X.$$

where M_X is the rate of mortality after X minutes under stress.

For formalin solution exposure stress testing, a test solution was prepared by mixing filtered seawater with 35-38 % formaldehyde solution (Wako Pure Chemical Industries, Japan) at 150 ppm concentration for larval and 200 ppm concentration for post-larval experiments

(Samocha et al. 1998). Then 10 test shrimp from each experimental tank were put in beakers (500 ml for larvae, 1 L for post-larvae) filled with test solution (100 ml for larvae, 200 ml for post-larvae) without supplying aeration. The time at which shrimp died after exposure to formalin solution was recorded to calculate the value of LT_{50}. LT_{50} is defined as the time required for 50 % mortality to occur due to a known stressor. This calculation was carried out as follows: the time to death was recorded and the survival values were converted to $\log_{10}$ (survival). These values of $\log_{10}$ (survival) were plotted against the time to the death to determine the duration of 50 % mortality of shrimp in each treatment. Each value obtained from the equation above was compared statistically. A higher value indicates greater tolerance against the stressors. Data was plotted following the linear equation $Y = aX + b$, where $Y = \log_{10}$ (survival), X = time to individual death of shrimp, and $LT_{50}(X)$ was obtained when $Y = 1.7$, since $\log_{10}(50) = 1.7$.

Biochemical Analysis

Proximate analysis of the diets and whole bodies of post-larvae were analyzed using standard AOAC methods (AOAC. 1990) In brief, this included removing moisture by oven-drying at 110 ℃ to a constant weight, crude protein measurement according to the Kjeldahl method, crude lipid measurement according to the Soxhlet extraction method, and ash measurement according to combustion in a Muffle furnace at 550 ℃ for 4 h.

Statistical Analysis

Statistical analyses were performed using analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). All data are presented as mean values standard ± error of mean (S.E.M., $n = 3$). Data from each group were compared using the Turkey Kramer Test. Differences between treatments were considered significant at $P < 0.05$.

Results

Larval Feeding Trial

Survival, growth parameters, number of larvae metamorphosed to the 1st post-larval stage, developmental stage, and tolerance against formalin solution exposure stress (LT_{50}) for larval shrimp are given in Table 1-2. All treatments showed survival rates between 81.3 % and 92 %. However, there were significant differences between the group fed 800 mg kg^{-1} level Ax and groups fed diets supplemented with Ax at 50, 100, and 200 mg kg^{-1} levels ($P < 0.05$). No significance differences were detected among the other groups ($P > 0.05$). The highest mortality occurred on the third day (about 5 - 10 %) during the 3rd zoea stage metamorphosis to 1st mysis stage. After this point, daily mortality was about 0-1 % for each group. The value of developmental (DS) and post-larval (PL) stages increased with increasing of dietary Ax from 0 to 200 mg kg^{-1} diet. Further, shrimp fed diets supplemented with Ax at 200, 400, and 800 mg kg^{-1} levels showed significantly higher DS than the control group ($P < 0.05$). The highest data of PL (93.9 %) and DS (2.94) was found at the 200 mg kg^{-1} supplemented level. Total body length (TL) was significantly higher in the group supplemented with 200 mg kg^{-1} level than in groups supplemented with both 0 and 800 mg kg^{-1} supplementation levels ($P < 0.05$), while no significances were detected among the other groups. In the formalin exposure stress test, the LT_{50} in the 50 and 100 mg kg^{-1} supplemented groups were significantly higher than in other groups ($P < 0.05$). However, shrimp fed with 800 mg kg^{-1} level Ax group exhibited the lowest value ($P > 0.05$).

Broken-line regression (Moe et al. 2005) was used to estimate optimum dietary Ax for growth (Fig. 1-1) and stress resistance (Fig. 1-2) parameters. A break-point at 168.9 mg kg^{-1} level Ax was estimated using the regression equation $Y = 0.0015x + 5.824$ ($R^2 = 0.99$) and $Y = -0.0004x + 6.145$ ($R^2 = 0.68$) on the total length measurement. A break-point at 82.1 mg kg^{-1} level Ax was estimated using the regression equation $Y = 0.09x + 34.5$ ($R^2 = 0.95$) and $Y = -0.0262x + 44.043$ ($R^2 = 0.93$) for formalin resistance.

Post-larval Feeding Trial

The results of survival (SR), final body weight (FBW), body weight gain (BWG), specific growth rate (SGR) and the tolerance against formalin exposure stress (LT_{50}) for the post-larval kuruma shrimp after the 30-day feeding trial are given in Table 1-3. All groups showed high survival rates, between 85.3 % and 92 %, with no significant differences among the experimental groups. FBW, BWG and SGR values were significantly higher at the 100 and 200 mg kg^{-1} supplementation levels than in the control group ($P < 0.05$), while no significant differences were detected among the other groups ($P > 0.05$). In the formalin exposure test (LT_{50}), shrimp fed diets containing Ax groups showed higher performance than the control group, with the highest performance observed in the 200 mg kg^{-1} level Ax group. No significance differences were detected between the other groups.

Whole body proximate composition of post-larval kuruma shrimp fed diets supplemented with different levels of Ax is shown in Table 1-4. There were no significant differences among groups ($P > 0.05$).

Cumulative mortality index (CMI) for osmotic stress is reported in Fig. 1-3. Shrimp fed Ax supplementation showed significantly lower values than the control group ($P < 0.05$), with the lowest value in the 200 mg kg^{-1} level Ax group.

Broken-line regression (Moe et al. 2005) was used to estimate optimum dietary Ax level on the growth (Fig. 1-4) and stress resistance (Fig. 1-5) parameters. A break-point at 108.7 mg kg^{-1} level Ax was estimated by the regression equation $Y = 0.985x + 407.67$ ($R^2 = 0.98$) and $Y = -0.0447x + 516.7$ ($R^2 = 0.83$) for body weight gain. A break-point at 178.1 mg kg^{-1} level Ax was estimated by the regression equation $Y = 0.0083x + 1.818$ ($R^2 = 0.94$) and $Y = -0.0019x + 3.635$ ($R^2 = 0.7$) for formalin resistance.

Discussion

Carotenoid compounds in aquaculture are well known to serve as a source of pro-vitamin A and to improve pigmentation (Miki et al. 1982; Latscha 1989). Many pure carotenoids, including β-carotene, echinenone, canthaxanthin, and zeaxanthin and crude carotenoids,

including oleoresin paprika, crayfish waste extract, corn gluten, alfalfa and spirulina, have been shown to improve pigmentation in crustaceans (Tanaka et al. 1976). Kuruma shrimp can transform certain dietary carotenoids into Ax (Tanaka et al. 1976). Moreover, based on comparison tests (Yamada et al. 1990, Chien and Jeng 1992), Ax appears to be the most effective pigment for kuruma shrimp. Many modern studies have used Ax supplementation in the form of Carophyll Pink®, a synthetic Ax with a free Ax molecule. Free Ax molecules were originally fat-soluble, but were altered to be water-soluble and coated with a gelatin matrix for improved bioavailability (Baker et al. 2002; Gouveia et al. 2003). Free Ax is the dominant carotenoid in the mature ovary, which suggests that it may play an important role in the development of eggs and larvae (Linan-Cabello et al. 2002).

Carotenoids are also known to play a positive role in the intermediary metabolism of aquatic animals (Segner et al. 1989). Carotenoids can enhance the utilization of other nutrients and may ultimately result in improved growth and reproductive potential (Mantiri et al. 1995; Petit et al. 1997; Amar et al. 2001). Our study supports these inferences, with Ax supplementation improving observed growth parameters. As a matter of fact, compared to controls, Ax-supplemented groups showed improved TL during the larval test, and improved FBW, BWG and SGR during the post-larval test. These results are consistent with previous studies finding that Ax supplementation can improve the growth of shrimp during the post-larval stage (Dall et al. 1995; Thongrod et al. 1995; Pan et al. 2001; Niu et al. 2009). In the present two studies, there were no significant differences in growth parameters detected among the Ax-supplemented groups. Although growth performance data rose as Ax content increased from 0 to 200 mg kg^{-1} diet, further increases beyond this point did not yield additional improvement. Both the highest growth data for larval and post-larval tests appeared at 200 mg kg^{-1} Ax supplementation, but the 100 mg kg^{-1} group in post-larval test also showed significantly higher growth performance than did the control group. Furthermore, the broken-line regression analyses indicated that the optimal level of growth performance for larvae (168.9 mg kg^{-1}) was higher than the level for post-larvae (108.7 mg kg^{-1}). Based on these analyses, we suggest that there may be a limited ability to metabolize dietary Ax that increases with shrimp growth. This

is supported by Petit et al. (1998) who reported that one-month post-larval shrimp are better able than earlier stages to absorb dietary pigments.

Initially, survival rate was considered the most important parameter in both the larval and post-larval experiments. However, we detected no positive correlation between Ax content in test diets and survival rate. These results are consistent with the findings reported by Chien and Jeng (1992), Boonyaratpalin et al. (2001), and Ju et al. (2011), who also found that Ax supplementation did not significantly affect shrimp survival. However, other authors have found contradictory results. Thongrod et al. (1995) suggested that increasing dietary Ax level from 0, 5, 15, and 60 to 300 mg kg^{-1} increased survival of post-larval *Penaeus monodon* during a 30-day feeding. However, Pan et al. (2001) found that the survival of post-larvae fed with Ax was improved only in the first week. Variation in survival effect may be related to light regime, species of shrimp, developmental stage, metabolic capacity, sources of Ax, or rearing conditions. The metabolic structure of larval shrimp may be sufficiently sensitive to be easily influenced by environmental factors.

Carotenoids such as Ax and β-carotene have also been reported to modify exuviation frequency, shorten the molting cycle, and hasten post-larval development (Dall et al. 1995; Petit et al. 1997). Our study showed higher values of DS and PL in all Ax-supplemented larval groups compared to controls. We suggest that both post-larval development and larval development speed could be increased under dietary Ax supplementation. Notably, neither DS nor PL improved at supplementation levels higher than 200 mg kg^{-1} level. This phenomenon may be also related to the metabolic capacity of different development stages. Taken together, our results and those of previous investigations suggest that Ax is an effective nutritional supplement for larval kuruma shrimp, with the quantities suggested in this study serving as a good starting point for further investigation into Ax metabolism and physiology.

Ax antioxidant activity has been reported as the strongest of the pure carotenoids (Shimidzu et al. 1996). In aquaculture, these properties can be closely associated with stress resistance (Chien et al. 2003). Acute stress tests are useful in defining larval and post-larval quality and can be used to distinguish between healthy and weak livestock (Samocha et al. 1998). In our study, freshwater osmotic stress and formalin exposure tests were conducted after feeding trials. LT_{50} and CMI results in a post-larval test suggested that dietary supplemented

Ax played a positive role in the acute stress resistance. This result is consistent with the reports by Merchie et al. (1998) and Darachai et al. (1998). Although the mechanism for improved osmotic tolerance under Ax supplementation has not been clarified, Darachai et al. (1998) concluded that Ax seemed to prolong the life of the post-larvae subsequent to acute stress.

Stress tolerance, growth, and development in larvae and post-larvae fed higher Ax supplementation (400 and 800 mg kg^{-1}) were not considerably better than that in groups under lower levels of supplementation (50, 100 and 200 mg kg^{-1}). Additionally, shrimp under 800 mg kg^{-1} supplementation showed significantly poorer performance than either control or 200 mg kg^{-1} supplementation groups, especially in stress resistance parameters. This suggests that excessive supplementation may be detrimental to both growth performance and stress resistance.

In conclusion, correctly dosed Ax supplementation appears to be useful in improving development, growth performance, and stress tolerance of larval and post-larval kuruma shrimp. I suggest optimized dietary levels for larvae of 168.9 mg kg^{-1} for growth and 82.1 mg kg^{-1} for stress tolerance performance. For post-larvae, optimal levels are 108.7 mg kg^{-1} for growth and 178.1 mg kg^{-1} for stress tolerance performance. Notably, excessive supplementation may lead to the paradoxical effects on the growth performance and stress resistance for both larval and post-larval kuruma shrimp. Therefore, I suggest using care in supplementing diets for kuruma shrimp.

Table 1-1. Composition of micro-bound diets (MBD). [a]

Ingredients (g kg^{-1})	Ax supplementation level (mg kg^{-1} diet)					
	0	50	100	200	400	800

Casein[b]	500	500	500	500	500	500
Pollack liver oil[c]	50	50	50	50	50	50
Soybean lecithin[b]	40	40	40	40	40	40
Cholesterol[d]	10	10	10	10	10	10
L-arginine[d]	30	30	30	30	30	30
Vitamin mixture[e]	30	30	30	30	30	30
Mineral mixture[f]	60	60	60	60	60	60
α-Starch	50	50	50	50	50	50
Sucrose[d]	50	50	50	50	50	50
Glucose[d]	50	50	50	50	50	50
Glucosamine-HCl[d]	8	8	8	8	8	8
Sodium citrate[d]	3	3	3	3	3	3
Sodium succinate[d]	3	3	3	3	3	3
k-Carrageenan[g]	25	25	25	25	25	25
Ax[h]	0	0.625	1.25	2.5	5	10
α-Cellulose	91	90.38	89.75	88.5	86	81
Total	1000	1000	1000	1000	1000	1000

[a] Approximate composition of the diets (%) based on dry weight: crude protein 53.4 %; crude lipid 9.8 %; moisture 9.8 %, ash 5.5 %.

[b] Wako Pure Chemical Industries, Ltd., Osaka, Japan.

[c] Riken Vitamin, Tokyo, Japan.

[d] Nacalai Tesque, Kyoto, Japan.

[e] Vitamin mix (30 g kg^{-1} diet) (Vitamin A free): *p*-aminobenzoic acid 0.23 g, biotin 0.01 g, inositol 9.17 g, nicotinic acid 0.92 g, Ca-pantothenate 1.38 g, pyridoxine-HCl 0.28 g, riboflavin 0.18 g, thiamine-HCl 0.09 g, menadione 0.09 g, α-tocopherol 0.46 g, cyanocobalamine 0.002 g, calciferol 0.03 g, stay-C 3.4 g, folic acid 0.02 g, choline choride 13.75 g.

[f] Mineral mix (60g kg^{-1} diet): K_2HPO_4 13.98g, $Ca_3(PO_4)_2$ 19.11g, $MaSO_4.7H_2O$ 21.36g, $NaH_2PO_4.2H_2O$ 5.55g.

[g] Sigma-Aldrich, St. Louis, MO, USA.

[h] Ax: Carophyll Pink® containing 8 % astaxanthin made by DSM Nutrition, Japan.

Table 1-2. Growth parameters and stress tolerance of larval kuruma shrimp fed with experimental diets for 8 days.[1]

Parameters[2]	Ax supplementation level (mg kg^{-1} diet)					
	0	50	100	200	400	800
SR (%)	86.3±1.76^{ab}	92.0±1.16^{b}	89.3±1.86^{b}	90.0±1.00^{b}	87.0±1.73^{ab}	81.3±1.45^{a}
DS	2.89±0.03^{a}	2.90±0.07^{ab}	2.91±0.06^{abc}	2.94±0.08^{d}	2.93±0.03^{cd}	2.92±0.04^{bcd}
PL (%)	88.7±0.26^{a}	89.8±0.72^{ab}	90.6±0.63^{abc}	93.9±0.75^{d}	92.8±0.33^{cd}	91.8±0.38^{bcd}
TL (mm)[3]	5.82±0.01^{a}	5.91±0.06^{ab}	5.97±0.07^{ab}	6.13±0.05^{b}	5.91±0.05^{ab}	5.88±0.02^{a}
LT_{50} (min)[4]	33.9±0.75^{b}	40.2±0.86^{c}	42.9±0.53^{c}	35.8±0.78^{b}	35.5±0.66^{b}	22.7±0.69^{a}

[1] Data are expressed as mean ± S.E.M. from triplicate groups. Different superscript letters indicate significant differences ($P < 0.05$).

[2] Abbreviations are as follows: survival (SR), developmental stage (DS), number of larvae metamorphosed to post-larval stage (PL) and total body length (TL).

[3] Total body length for 10 larval kuruma shrimp.

[4] LT_{50} is defined as the time required for 50 % mortality to occur due to the formalin exposure stress.

Table 1-3. Growth parameters of post-larval kuruma shrimp fed with experimental diets for 30 days.[1]

Parameters[2]	Ax supplementation level (mg kg^{-1} diet)					
	0	50	100	200	400	800
SR (%)	85.3±1.45	92.0±1.16	90.0±1.53	87.3±1.33	86.7±1.76	85.7±1.45
FBW (mg)	$40±0.01^{a}$	$45±0.02^{ab}$	$48±0.02^{b}$	$49±0.02^{b}$	$47±0.01^{ab}$	$47±0.01^{ab}$
BWG (%)	$404.2±15.0^{a}$	$462.5±26.0^{ab}$	$500.0±21.7^{b}$	$512.5±19.1^{b}$	$491.7±18.2^{ab}$	$483.3±15.1^{ab}$
SGR(%/day)	$5.39±0.10^{a}$	$5.75±0.15^{ab}$	$5.97±0.12^{b}$	$6.04±0.10^{b}$	$5.92±0.10^{ab}$	$5.88±0.09^{ab}$
LT_{50} (h)[3]	$1.78±0.23^{a}$	$2.43±0.29^{ab}$	$2.43±0.17^{ab}$	$3.54±0.17^{b}$	$2.45±0.38^{ab}$	$2.26±0.48^{ab}$

[1] Data were expressed as mean ± S.E.M. from triplicate groups. Different superscript letters indicate significant differences ($P < 0.05$).

[2] Abbreviations are as follows: survival (SR, %) = (final number of shrimp/initial number shrimp) × 100; final body weight (FBW), body weight gain (BWG, %) = [(final weight-initial weight)/initial weight] × 100; specific growth rate (SGR, %/day) = [(ln final weight-ln initial weight)/duration] × 100

[3] LT_{50} is defined as the time required for 50 % mortality to occur due to the formalin exposure stress.

Table 1-4. Whole-body compositions (% dry weight) of post-larval kuruma shrimp fed with experimental diets for 30 days.[1]

Parameters	Ax supplementation level (mg kg^{-1} diet)					
	0	50	100	200	400	800
Crude protein	65.9±0.5	66.2±0.4	64.9±0.2	65.3±0.3	65.5±0.2	65.6±0.1
Crude lipid	14.7±0.2	14.4±0.1	14.8±0.2	15.1±0.1	14.8±0.1	15.0±0.1
Ash	21.3±0.1	21.2±0.2	21.0±0.3	21.3±0.4	21.4±0.5	21.1±0.2
Moisture	83.6±0.2	82.1±0.3	79.8±0.5	78.4±0.6	87.3±0.7	84.5±0.6

[1] Data are expressed as mean ± S.E.M. from triplicate groups.

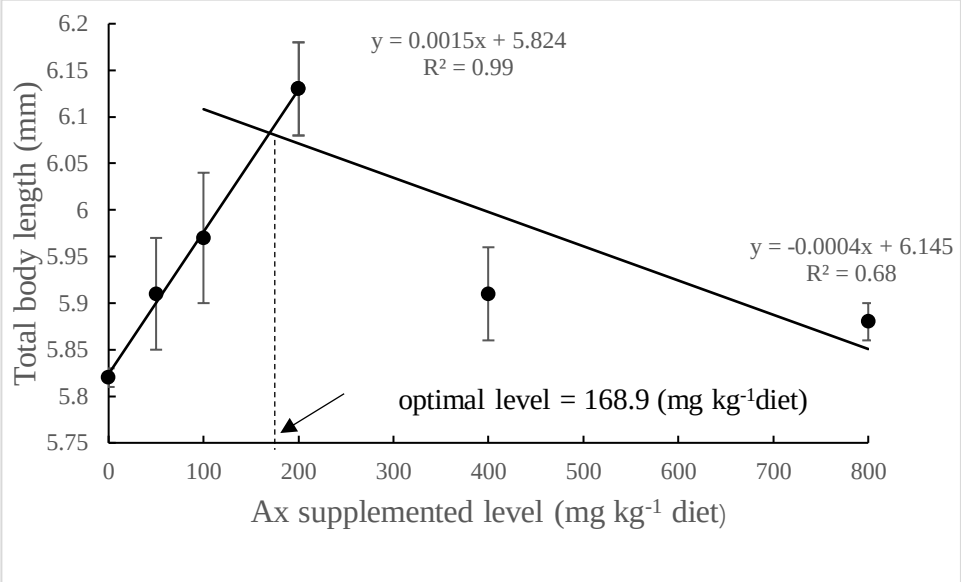

Figure 1-1. Broken-line analysis of total body length in larval kuruma shrimp with experimental diets for 8 days.

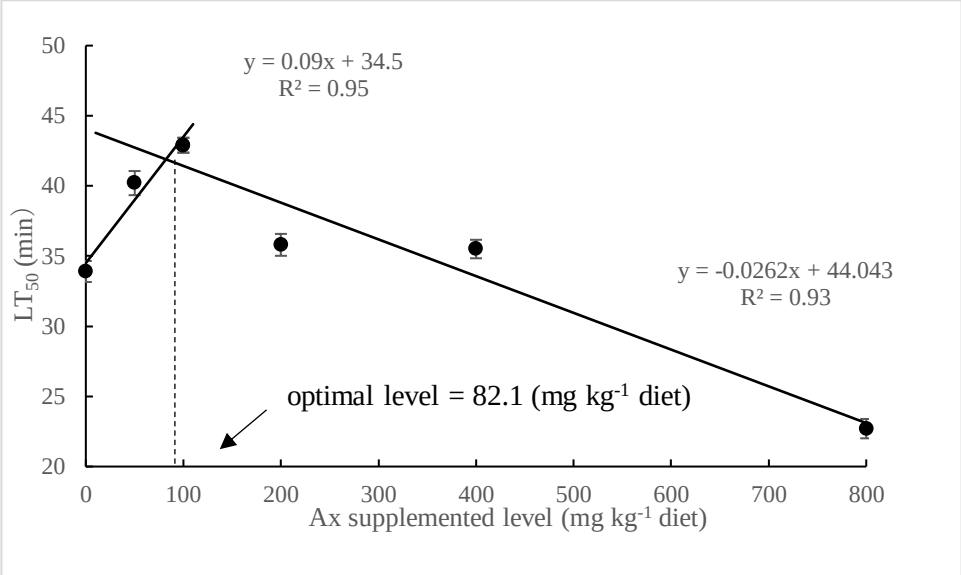

Figure 1-2. Broken-line analysis of time to 50 % mortality (min) of larval kuruma shrimp under formalin stress, after Ax supplementation for 8 days.

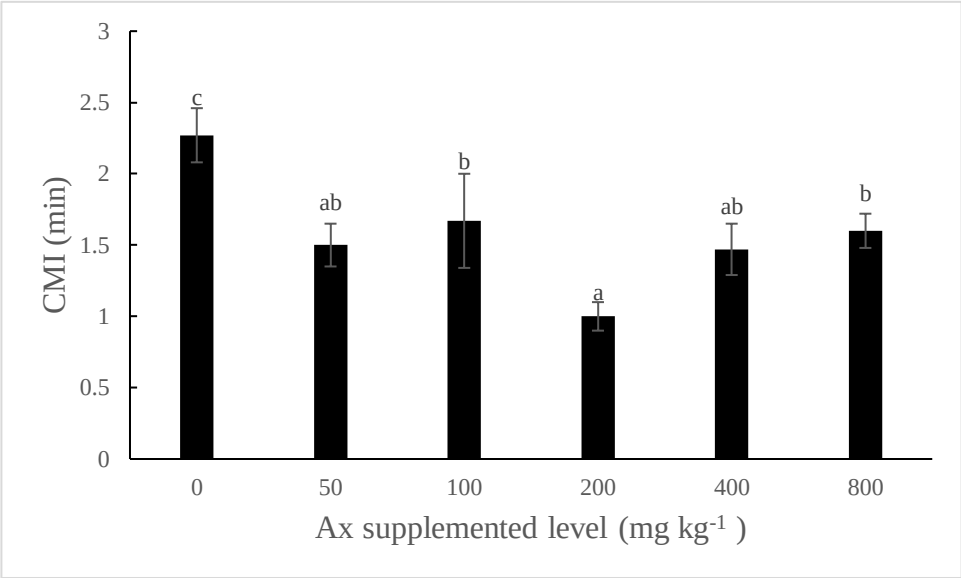

Figure 1-3. Cumulative mortality index (min) of post-larval kuruma shrimp under fresh water stress, after Ax supplementation for 30 days.

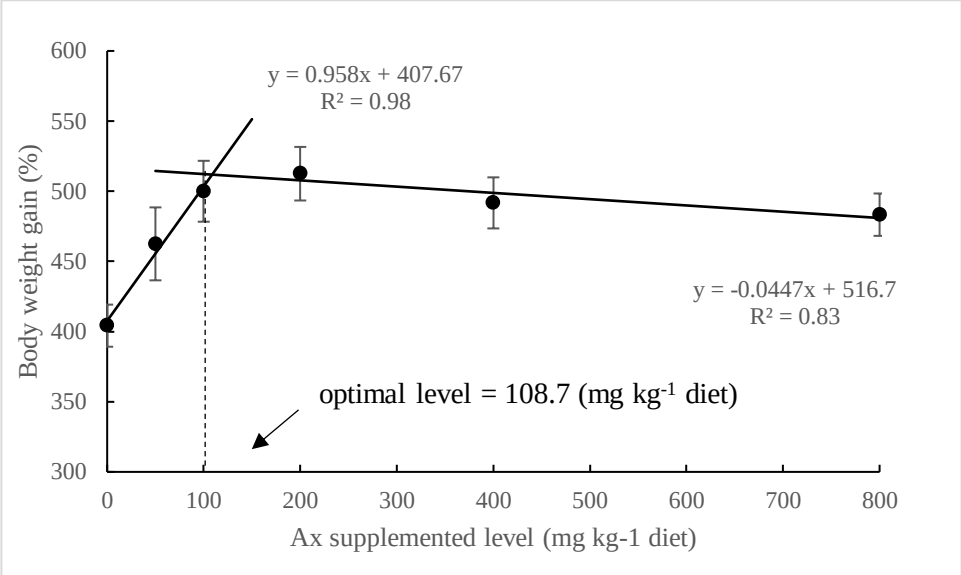

Figure 1-4. Broken-line analysis of body weight gain in post-larval kuruma shrimp fed with experimental diets for 30 days.

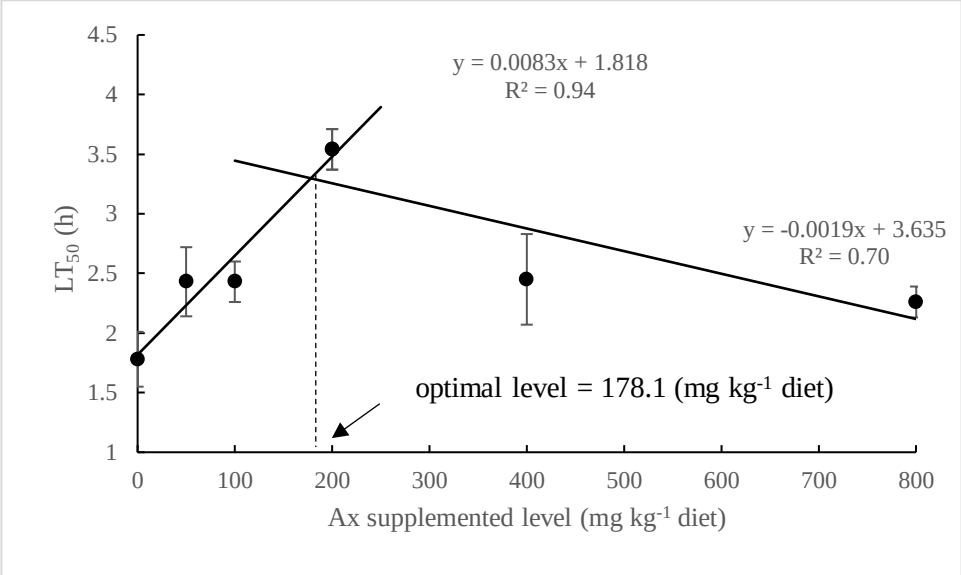

Figure 1-5. Broken-line analysis of time to 50 % mortality (h) after formalin stress testing of post-larval kuruma shrimp fed with experimental diets for 30 days.

Study 2: **Effects of Dietary Astaxanthin Supplementation on Juvenile Kuruma Shrimp, *Marsupenaeus japonicus***

Abstract

This study evaluated the effects of chemically synthesized astaxanthin (Ax) on growth performance, survival, stress resistance, immune responses, fatty acid contents, and pigmentation of juvenile kuruma shrimp (*Marsupenaeus japonicus*). Six dietary levels of Ax (0, 200, 400, 800, 1200, and 1600 mg kg^{-1} diet) were added to a baseline diet. The feeding trial was conducted for 56 days. The results showed that juveniles fed diets supplemented with Ax exhibited better growth performance, final body weight (FBW), body weight gain (BWG), and specific growth rate (SGR) than the control group, with the best performance exhibited by in the 400 mg kg^{-1} diet Ax supplemented group ($P < 0.05$). Following fresh water-osmotic stress test, the 200, 400, and 800 mg kg^{-1} diet Ax supplemented groups showed higher LT_{50} values than the other groups. The 400, 800, and 1200 mg kg^{-1} diet Ax supplemented groups exhibited different immune responses (total hemocyte count [THC] and viable cells [VC]) to the control group. The Ax content of the whole shrimp body increased with increasing supplemented levels. After cooking, shrimp fed diets containing Ax were a strong red in color, while the control group was light pink in color. The colorimetric reading of the body and head of cooked shrimp showed that redness (a^*) and yellowness (b^*) increased with increasing dietary Ax supplementation. The 1200 and 1600 mg kg^{-1} diet supplemented groups were also significantly different from the control group for these two parameters in both shrimp body and head. Broken-line regression analysis indicated that the optimal levels for growth, immune responses, and pigmentation were 401, 420, and 404 mg kg^{-1} diet, respectively. In conclusion, the optimum diet supplementation level of the Ax to enhance the performance of juvenile kuruma shrimp is around 400 mg kg^{-1}.

Keywords: kuruma shrimp, astaxanthin, pigmentation, immune response, optimum

Introduction

Kuruma shrimp, *Marsupenaeus japonicus*, is one of the most important crustacean species in Japan (Bulbul et al., 2014). However, kuruma shrimp catches have decreased almost linearly since 1980 in Japan (FAO, 2010). Therefore, aquaculture represents the only viable way of bridging the gap between the demand and supply of this high-value seafood. However, kuruma shrimp cultured under intensive culture conditions with artificial diets are less colorful than natural ones (Chien and Jeng, 1992).

In the shrimp culture industry, the color of shrimp flesh is an important criterion of quality that affects the choice of consumers in the marketplace (Lucien-Brun and Vidal, 2006). Thus, the degree of red color development is a very important factor determining the commercial value of shrimp (Niu et al., 2009). Therefore, it is necessary to add pigments to diets to enhance the color of shrimp and improve their commercial value.

Carotenoids pigments are widely present in aquatic animals. Crustaceans cannot biosynthesize carotenoids on their own, but are able to alter dietary carotenoids by oxidation, and deposit these in their tissues (Meyers and Latscha, 1997). The inclusion of pure carotenoids (β-carotene, echinenone, canthaxanthin and zeaxanthin) and crude carotenoids (oleoresin paprika, crayfish waste extract, corn gluten, alfalfa, and spirulina) in diets improves pigmentation in crustaceans through various biosynthetic processes, as described by Tanaka et al. (1976).

However, astaxanthin (Ax) is the most effective pigment (Chien and Jeng, 1992) because it is easily absorbed through the digestive tract and is preferentially deposited in the flesh (Torrissen, 1989). The pigmentation efficiency of dietary Ax depends on the solubility and bioavailability of the material and levels of Ax present (Mensaveta et al., 1993). Chemically synthesized pigment "Carophyll Pink®" is a free Ax molecule that is coated with a matrix of gelation to enhance its bioavailability and water-dispersible qualities. Therefore, Carophyll Pink® is generally used as the source of Ax in aqua-feeds.

In addition to pigmentation properties, Ax also has many positive biological effects for shrimp, such as enhancing maturation, increasing the immune response, and reducing stress associated with ammonia levels (Merchie et al., 1998; Pan et al., 2001; Chien et al., 2003). Ax is also important for animal health as an antioxidant, through the deactivation of free radicals produced from normal cellular activity and various stressors (Chew, 1995; Dose et al., 2016).

Previous studies have investigated optimum levels of synthetic Ax (Carophyll Pink®) for kuruma shrimp. Chien and Jeng, (1992) and Yamada et al. (1990) suggested optimum levels of 1000 and 200 mg kg^{-1} in the diet, respectively. However, the optimum levels of Ax supplementation in these studies was dependent on the biological function of Ax. Unfortunately, information remains limited on how dietary Ax affects the immune response, fatty acid content, and colorimetric color readings of this species. Therefore, the present study aimed to evaluate how dietary Ax affects the growth performance, survival, and stress resistance of juvenile kuruma shrimp, in addition to its immune response, fatty acid content, and pigmentation. Our results are expected to provide baseline information on the optimum level of Ax supplementation for kuruma shrimp under aquaculture conditions.

Materials and Methods

Preparation of Astaxanthin

A chemically synthesized pigment (Carophyll Pink®) containing 8% Ax made by DSM Nutrition, Japan was used for the experiment. Because of the relatively large differences in the amount of Ax and other carotenoids in this source, the contribution of other carotenoids was not considered.

Shrimp and Experimental System

Kuruma shrimp, *M. japonicus*, was obtained from Matsumoto, Miyazaki, Japan, and transported to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University. The shrimp was maintained at an ambient water temperature (23 ± 0.5°C) in a 500-L black polyethylene circular tank. After acclimatization to laboratory conditions (i.e., 1 week), 10 kuruma shrimp juveniles (1.41 ± 0.01 g initial wet weight) were randomly selected from the stock tanks and were transferred to the experimental tanks for the feeding trial. The feeding trial was conducted in eighteen 54-L rectangular polyvinyl chloride (PVC) tanks filled with

45-L seawater. Sand (approximately 20 mm thickness) was placed on the bottom of each tank. Throughout the experiment, the tanks were continuously aerated, while the salinity of the rearing water was maintained at 34 ± 0.5 ppt, with a flow rate of 60 mL min^{-1}. Each tank was covered with a net and black plastic film to prevent the shrimp from jumping out and to minimize disturbances.

Six iso-nitrogenous, iso-lipidic and iso-caloric pellet diets were prepared following the method described by Tung et al. (2010). The lipid sources and fat-soluble components were premixed and then added to the dry ingredients until mixed well. Then, water was added to the mixture at a quantity of 35-40% of the dry ingredients, and the pH was adjusted to 7.0-7.5 using 1N sodium hydroxide. The mixture was then molded into an appropriate diameter (1.2-2.2 mm) to the prepare pellets using a single-screwed mincer (ROYAL Inc., Tokyo, Japan). The pellets were then dried in a dryer mechanical convection oven (DK400, Yamato Scientific, Tokyo, Japan) at 60°C until the moisture content was reduced to 10%. The dried pellets were steamed at 100°C for 1 min in a cylindrical steamer to improve their water stability, and were stored at -20°C until use.

The basal composition of the test diets is shown in Table 2-1. Casein was used as the major protein source. *L*-arginine was added to meet the amino acid requirement of this species according to Teshima et al. (1986). *a*-Starch was used as the carbohydrate source. Pollack liver oil and cholesterol were used as the major lipid sources, while, soybean lecithin was used as the phospholipids source. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were also added to the diet to provide a source of n-3 polyunsaturated fatty acid (PUFA). The mineral and vitamin mixtures were added according to Moe et al. (2004) and Alam et al. (2004) with slight modifications. Vitamin A was not added, so that supplemented Ax was available and represented the single variable. Glucosamine-HCl, sodium succinate and sodium citrate were also added as attractants. Other ingredients, such as sucrose and glucose, were also added to meet the growth requirements for juvenile shrimp. Six levels of Ax supplements were added to the diets: 0 (control), 200, 400, 800, 1200, and 1600 mg kg^{-1} diet. Carboxymethyl-cellulose (CMC) was added to pre-coat CAA and Ax to prevent loss through leaching. *k*-Carrageenan was added to the mixture to improve water stability of the pellet diets. The biochemical analysis of the diet is shown in Table 2-2.

Feeding Trial of Juveniles

Juveniles were fed twice daily with test diets at a ration amount of 8-10% of body weight. Twenty-percent of the daily ration was provided at 08:00 and 80% at 17:00. Each morning, uneaten food was collected, while fecal matters was siphoned from the tank. All uneaten food was collected and freeze-dried to calculate feed intake and the feed efficiency ratio. Every 10 days, juveniles were bulk-weighted, and the survivors in each tank were counted. At the same time, the tank and sand were cleaned and filled with fresh rearing water.

Total Hemocyte Count, Viable Cells, and Phagocytic Activity Assay

The total hemocyte count (THC), viable cells (VC), and the rate of phagocyte activity (PA) were assayed as indicators for the immunological assay. The method described by Itami et al. (1998) was used with slight modification. A quantity of 200 µL hemolymph was collected from the ventral sinus of shrimp with 800 µL of sterile anticoagulant (NaCl 29.22 g L^{-1}, EGTA 3.8 g L^{-1}, Hepes 2.38 g L^{-1}, and L-cysteine 5 g L^{-1} of stock solution) using a 1-mL syringe. The mixture was then centrifuged at 1000 rpm and 5°C for 10min (MX160, Tomy, Tokyo, Japan). The supernatant was discarded and the pellet was washed three times with 1 mL shrimp saline solution (NaCl 28.4 g L^{-1}, $MgCl{\cdot}6H_2O$ 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 2 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 2.25 g L^{-1}, KCl 0.7 g L^{-1}, Glucose 1 g L^{-1}, Hepes 2.38 g L^{-1}) by centrifugation. The sediment was re-suspended in shrimp saline solution and separated into two equal sub-samples; one sample was used to measure the THC and VC, the other sample was used to measure PA. To count THC and VC, the diluted hemocytes were mixed with equal volume of trypan blue using a hemocytometer (Burker-Turk, Minato Medical Corporation, Tokyo, Japan). VC was calculated as:

$$\text{Viable Cell (\%)} = \text{Non-stained cells/total hemocytes} \times 100$$

To quantify phagocytic activity, 200 µL hemocytes at a concentration of 1×10^6 cells mL^{-1} were spread on a cover slip (size 22 × 24 × 0.4 mm) in six well-plates with a cover and were mixed with heat-killed yeast (5×10^8 cells mL^{-1}). After incubation at 25°C for 2 h, the samples were fixed by Fixative (4% paraformaldehyde in 0.2 M sodium cacodylate buffer with 10% sucrose). The samples were then stained with Wright solution (Wako Pure Chemical Industries, Ltd, Japan) and mounted with Eukitt (Entellan, Merck, Germany) for the yeast method. The number of phagocytosis cells out of 200 cells was counted, and the rate of phagocyte activity (PA) was calculated as:

$$PA\ (\%) = \text{Phagocytosis cells/total hemocytes} \times 100$$

Astaxanthin Analysis

The shrimp samples were freeze dried and then crushed to form a powder. The samples were then placed into a 50-mL polypropylene centrifuge tube for Ax analysis following the method of Masako et al. (2010), with slight modifications. The Ax was then extracted with chloroform by ultrasonic mixing for 10 min and was centrifuged at 4°C and at 11800 rpm for 15 min. The extracted Ax was transferred to flasks and dried in rotary vacuum evaporator (Eyela SB 1100, CCA 1111). The sample extract was then dissolved in 10 mL acetone solution. A volume of 0.05 M Tris-HCl buffer (pH 7.0) 6 mL and cholesterol esterase solution (60-unit mL^{-1}) 600 µL was added to the extract, and the enzyme reaction allowed to complete for 120 min at 37°C. After the enzyme reaction, the solution was transferred to a 250-mL separatory funnel, partitioned with 10 mL petroleum ether. The solution was washed three times with 0.01 g $NaSO_4$ to remove residual acetone. Then, using the rotary vacuum evaporator again, the solvent was mobilized and filtered through a 0.2-µm Millipore filter and stored in brown vials. Ax was analyzed by high-performance liquid chromatography (HPLC), using a shim-pack VP-ODS column (150 mmL. × 4.6 mmL.D.) and a SPD-M20A detector at 475 nm. The operational conditions were: mobile phase, 0.05% trifluoracetic acid/methanol = 3/97 (v/v) and solvent flow rate, 1.2 mL min^{-1}; injection volume, 10 µL. This system was controlled by a chromatographic data system (Scientific Information Services Corporation), which also

integrated the areas under the peaks. The standard was prepared for five levels (0.05-20 mg L^{-1}) using 8% Ax contained in Carophyll Pink®. And the standard curve was presented by the equation Y = 55784x - 11087 (R^2 = 0.99).

Fatty Acid Analysis

Fatty acid analysis was analyzed using a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector using an Omegawax 320 column (Supelco, Inc., Japan). FA methyl esters were prepared for the shrimp body according to the method of Bligh and Dyer (1959) and then further separated into neutral lipid (NL) and polar lipid (PL) fractions by Sep-pak silica cartridge (Waters Corporation, Milford, Massachuseetes, USA). Methylating the NL and PL by adding C23:0, respectively. And keeping the samples into the heat reactor for 1 h with boron trifluoride and dichloromethane. After that, mixing with NaCl and hexane, and taking the upper layer for further fatty acid analysis. The relative quantity of each FA present was determined by measuring the area under the chromatograph peak corresponding to that fatty acid.

Color Reading

The plastic bags of samples (six shrimp/bag) were placed in a boiling water bath for 3 min. The bags were then cooled in tap water (about 5°C) in the dark following the method of Ju et al. (2011), with slight modifications. And Ando et al. (2014) stated that kuruma shrimp cooked at 100°C and stored at 5°C showed better color development rate. Color parameters (L^* = lightness, a^* = redness, b^* = yellowness) were obtained with a colorimeter (Chroma Meter CR400, Konica Minolta Sensing Inc., Osaka, Japan) according to Nickell and Bromage (1998). Three parts including head, body and muscle (shell removed) of shrimp were read by the colorimeter.

Stress Resistance Test

Fresh water-osmotic stress tests were conducted after the feeding trial. Shrimp were challenged with low salinity conditions to investigate their tolerance against stressors and to evaluate the efficiency of test diets. Five test shrimp from each experimental tank were placed in 10-L glass bottles filled with 8-L tap water that was dechlorinated by aeration for 24 h before starting the stress test. Mortality was monitored every 10 min. Survival was expressed as the LT_{50}. LT_{50} was defined that as the time required for 50% mortality of the test shrimp by stressors. The time to death (min) was recorded and the survival values were converted to $\log_{10}$(survival). These $\log_{10}$ (survival) were plotted against the time to the death to determine the time required for 50% mortality of shrimp in each treatment. Each value obtained from the equation above was compared statistically. The higher value indicated the greater tolerance against the stressors. The equation was presented as:

$$Y = aX + b$$

where $Y = \log_{10}$(survival), X = time to the death of individual shrimp. $LT_{50}(X)$ was obtained when $Y = 1.7$, because $\log_{10}(50) = 1.7$

Biochemical Analysis

Proximate analysis of the diets and the whole body of shrimp were analyzed by standard AOAC methods (AOAC, 1990). Moisture content was determined by oven-drying at 110°C to constant weight. Crude protein content was determined by the Kjeldahl method, crude lipid content by the Soxhlet extraction method, and ash content by combustion in a Muffle furnace at 550°C for 4 h.

Statistical Analysis

Statistical analysis was performed using an analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). All data are presented as mean ± standard error of mean (S.E.M., n = 3). Data from each group were compared using Duncan's new multiple range test. Differences between treatments were considered significant at $P < 0.05$.

Results

The survival rate (SR), initial body weight (IBW), final body weight (FBW), body weight gain (BWG), specific growth rate (SGR) and feed efficiency (FE) for the juvenile kuruma shrimp after the 56-day feeding trial are shown in Table 2-3. All groups had similar SR of around 62%, with no significant differences among the experimental groups. FBW, BWG, and SGR showed significantly higher performances ($P < 0.05$) in the 400 and 800 mg kg^{-1} diet Ax supplemented group compared to the control group. Other supplemented groups exhibited intermediate performance. The control group (no supplement) showed the lowest growth performances values. The feed efficiency ratio was numerically higher in the supplemented groups, but was not significantly different to any of the Ax supplementation groups.

The proximate compositions of the bodies of juvenile kuruma shrimp fed diets supplemented with different levels of Ax are shown in Table 2-4. The compositions of all shrimp showed some degree of change in the analyzed parameters to the initial values. However, there were no significant differences ($P > 0.05$) in the final proximate compositions of whole shrimp bodies among all groups of shrimp fed the different experimental diets.

The LT_{50} values for the fresh water stress tolerance test are shown in Fig. 2-1. The LT_{50} values were significantly influenced by the dietary supplementation of Ax, increasing ($P < 0.05$) up to the 800 mg kg^{-1} diet Ax supplemented group, after which the LT_{50} values decreased. However, there were no significant differences among the 200, 400, and 800 mg kg^{-1} diet Ax supplemented groups. The lowest value was obtained in the control group; this was not significantly different from the ≥1200 mg kg^{-1} diet Ax supplemented groups.

The immune response results of THC, VC, and PA for the juvenile shrimp after the 56 days feeding trial are shown in Fig. 2-2 and Fig. 2-3. The shrimp groups that received Ax supplemented diets had higher PA, HTC, and VC values than the control group. PA was significantly higher in the 1200 mg kg^{-1} diet Ax supplemented group, but did not significantly differ ($P > 0.05$) to the 400 and 800 mg kg^{-1} diet Ax supplemented groups. The 1600 mg kg^{-1} diet Ax supplemented group had an intermediate PA value, while ≤ the 200 mg kg^{-1} diet Ax

supplemented groups had significantly lower PA values. THC values increased significantly with increasing level of Ax supplementation, with higher THC values being obtained in the 1600 mg kg^{-1} diet Ax supplemented group; however, the THC values of this group did not significantly differ from those in the ≥400 mg kg^{-1} diet Ax supplemented groups or the 200 mg kg^{-1} diet Ax supplemented group. Numerically higher VC values were obtained in all supplemented groups, whereas VC did not significantly differ among treatments.

The fatty acid profiles in the shrimp bodies are shown in Table 2-5. Compared to the control, the EPA of neutral lipids was significantly higher in the 800 mg kg^{-1} diet Ax supplemented group, followed by the 1600, 1200, 400, and 200 mg kg^{-1} diet groups, respectively. Compared to the control, the DHA of neutral lipids was also significantly higher in the 800 mg kg^{-1} diet Ax supplemented group, but did not differ significantly with the 1200 mg kg^{-1} diet or the 1600 mg kg^{-1} diet Ax supplemented groups. Diet Ax supplemented groups with ≤400 mg kg^{-1} did not significantly differ from the control group. The EPA of polar lipids increased with increasing Ax supplementation, and was significantly higher in the 400 mg kg^{-1} diet Ax supplemented group, followed by the 1600, 1200, 200, and 800 mg kg^{-1} diet Ax supplemented groups. The DHA values of polar lipids also increased with increasing Ax supplementation, but did not significantly differ among dietary treatments.

The total Ax content of the diet and shrimp whole body are showed in Table 2-6. The results show that the determination contents of Ax in the diet were similar to the supplementation levels. Furthermore, the Ax content of the shrimp whole body increased with increasing Ax supplementation level.

The colorimetric effect of Ax on experimental shrimp heads and bodies measured using the three color parameters (L^*, a^*, b^*) are shown in Table 2-7. Lightness value (L^*) was less affected by Ax supplementation, whereas redness (a^*) and yellowness (b^*) increased with increasing Ax supplementation. Furthermore, for the head part of shrimp, the 400, 800, 1200, and 1600 mg kg^{-1} diet Ax supplemented groups had significantly higher redness (a^*) and yellowness (b^*) values than the control group. However, there were no significant differences among these four groups. A significantly lower colorimetric reading of redness (a^*) and yellowness (b^*) was obtained in the control group when compared to the 1200 and 1600 mg kg^{-1} diet Ax supplemented groups on shrimp bodies.

Broken-line regression (Moe et al., 2005) was used to estimate the optimum dietary Ax level in relation to growth performance (Fig. 2-4), immune response (Fig. 2-5), and pigmentation (Fig. 2-6). A break-point at 401 mg kg^{-1} diet level Ax was estimated using the regression equation $Y = 0.0308x + 42.17$ ($R^2 = 0.96$) and $Y = -0.0047x + 56.42$ ($R^2 = 0.83$) on the body weight gain measurement. A break-point at 420 mg kg^{-1} diet level Ax was estimated using the regression equation $Y = 0.0108x + 5.055$ ($R^2 = 0.99$) and $Y = 0.0022x + 8.665$ ($R^2 = 0.97$) on the total hemocyte count measurement. A break-point at 404 mg kg^{-1} diet level Ax was estimated using the regression equation $Y = 0.009x + 11.055$ ($R^2 = 0.96$) and $Y = 0.0011x + 14.25$ ($R^2 = 0.79$) on the redness (a^*) measurement of shrimp body.

Discussion

Carotenoids have a positive role on the intermediary metabolism of aquatic animals and might enhance nutrient utilization leading to improved growth (Segner et al., 1989; Amar et al., 2001). The results of the present supported this hypothesis, based on the FBW, BWG, and SGR values after the 56-days feeding trial. All the groups fed with supplemented Ax performed better than the control group. These results also support those of Darachai et al. (1998) and Niu et al. (2012). Our results showed that the 400 mg kg^{-1} diet Ax supplementation level had the highest values that were significantly different to the control and 200 mg kg^{-1} diet Ax supplemented groups. Therefore, supplementation with low levels of Ax (200 mg kg^{-1} diet) minimally impacted the growth parameters of shrimp. Furthermore, shrimp growth did not significantly increase above 400 mg kg^{-1} diet supplementation. Thus, the 400 mg kg^{-1} diet supplementation level might be close to the absorption tripping point of the digestive tract for juvenile shrimp feeding on Ax.

After the feeding trial, no significant differences ($P > 0.05$) were found among treatments regarding survival. Ju et al. (2011), Chien and Jeng (1992) and Boonyaratpalin et al. (2001) also found that Ax supplementation did not significantly affect shrimp survival. This result might have been obtained because the seawater temperature (23 ± 0. 5°C) during the feeding period in October and November of the current study was lower than the optimal growth

temperature for this species. Consequently, the feed intake of each group was lower than the expected value. Moreover, it was difficult to collect uneaten food; thus, the feed efficiency did not show clear differences among the groups in the data analysis.

The lethal stress test was used to assess the health status by measuring the stress tolerance of the experimental shrimp. The results of the present study showed that Ax supplemented groups performed better than the control at LT_{50}. Recent studies also showed that the resistance to oxygen depletion stress (Chien et al., 1999), salinity stress (Darachai et al., 1998), thermal stress (Chien et al., 2003), and ammonia stress (Pan et al., 2003) was enhanced in penaeid shrimp associated with higher dietary Ax. Thus, Ax might represent a favorable factor when shrimp are subject to physiological stress caused by abiotic changes. Previous studies inferred this phenomenon, despite lacking biochemical evidence. Chien et al. (1999) suggested that Ax serves as an intracellular oxygen supply for shrimp, allowing survival under the hypoxic conditions at the pond bottom. Craik (1985) suggested that Ax acts as an intracellular oxygen reserve for respiration in salmonid eggs subjected to oxygen, in which oxygen is attached to the center of the hydrocarbon chain. However, while many studies have suggested high tolerance to low dissolved oxygen, information on lethal stress remains rare. The ideal antioxidant properties of Ax might be closely associated with stress resistance (Torissen, 1989; Shimidzu et al., 1996). Resistance to fresh water was significantly enhanced in the 200, 400, and 800 mg kg^{-1} diet supplemented groups. This phenomenon might also explain the absorption tripping point of Ax in this species, as recorded for growth parameters.

Blood parameters are considered a reliable indicator of the health status and physiological conditions of shrimp (Itami et al., 1998). The performance of THC, VC and PA in the present study was higher in the groups fed with the Ax supplemented diet. However, an exponential increase was not obtained with increasing Ax supplementation level. The 400, 800, 1200, and 1600 mg kg^{-1} diet Ax supplemented level groups significantly differed ($P < 0.05$) to the control for THC and PA, with no significant difference being detected among these four levels ($P > 0.05$). The performance of the 200 mg kg^{-1} diet Ax supplemented group was similar to that of the control. Thus, low dietary Ax does not significantly affect the blood parameters of juvenile shrimp. Bachere (2000) stated that the immune response must be analyzed under stress conditions in cultured shrimp. Tung et al. (2010) also suggested that, under conditions of

salinity stress, blood parameter measures were sufficiently different. Future studies should obtain values for both conditions to enhance the results.

The visual inspection of cooked whole shrimp bodies clearly showed that shrimp fed the with the Ax supplemented diet had a more pronounced red color than the light pink color of the control group. Our results were consistent with the findings of Ju et al. (2011). Similar results were observed on both the head and body part of the same shrimp. Our results of the parameters measured using colorimeter show that either redness (a^*) or yellowness (b^*) increased with increasing dietary Ax supplementation. The yellowness and redness of the head of shrimp from the 400, 800, 1200, and 1600 mg kg^{-1} diet Ax supplemented level groups were significantly different ($P < 0.05$) to the control. Redness of shrimp body from the 800 mg kg^{-1} diet to 1600 mg kg^{-1} diet supplemented groups was significantly different ($P < 0.05$) to the control, while the yellowness was significantly different for the 1200 mg kg^{-1} diet to 1600 mg kg^{-1} diet Ax supplemented groups. Yanar et al. (2012) showed that the total carotenoid content of the head part of kuruma shrimp was higher than that of the body shell and muscle. Thus, the head part of shrimp exhibits higher pigmentation efficiency after animals are fed with Ax supplemented diets. However, no significant difference was observed among the Ax supplemented groups (400, 800, 1200, and 1600 mg kg^{-1} diet) for these two types of pigmentation on either the head or body part of shrimp. The results of the present show that redness (a^*) parameters increased with increasing Ax supplementation, supporting our results on whole body Ax concentrations.

Previous studies on lipid nutrition demonstrated that PUFAs are the dominant fatty acids in marine animals. Kanazawa et al. (1979) reported that *M. japonicus* has a limited ability for the de novo synthesis of PUFAs, such as cosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA), which have higher activity. Increased levels of dietary carotenoids lead to the accumulation of carotenoid esters in crustaceans (Yamada et al., 1990; Boonyaratpalin et al., 2001). Wade et al. (2017) also showed that Ax monoesters are enriched with saturated fatty acids, whereas Ax diesters are enriched with monounsaturated and polyunsaturated fatty acids. Consistent with these findings, our results showed that EPA and DHA increase with increasing Ax supplementation. However, the esterification of Ax with specific fatty acids and the presence of carotenoid isomers might significantly increase the complexity of the interaction

between carotenoids and other biological molecules or membranes (Goodwin, 1986; Britton, 1995). Future studies should focus on identifying the specific carotenoid fatty acid esters that accumulate in the tissues of kuruma shrimp, which might regulate the positive effects of carotenoids.

The present study showed that Ax supplementation enhances the pigmentation of juvenile kuruma shrimp, in addition to enhancing their immune response, stress resistance response, growth performance, and fatty acid content. We showed that supplementation with around 400 mg kg^{-1} dietary Ax produced the best growth performance and stress resistance responses. Furthermore, the immune response, color parameters, and EPA content significantly differed from the control above 400 mg kg^{-1} Ax dietary supplementation. Overall, the optimum dietary supplementation level of Ax (Carophyll Pink®) for juvenile kuruma shrimp was around 400 mg kg^{-1}. In conclusion, my results demonstrate the potential economic benefits of Ax supplementation in kuruma shrimp aquaculture systems, through improved growth performance, and enhanced pigmentation and immune responses.

Table 2-1. Composition of pellet diets.

Ingredients (g kg^{-1})	Ax supplementation level (mg kg^{-1} diet)					
	0	200	400	800	1200	1600
Casein[a]	500	500	500	500	500	500
Pollack liver oil[b]	50	50	50	50	50	50
Soybean lecithin[a]	32	32	32	32	32	32
PUFA[d]	8	8	8	8	8	8
Cholesterol[c]	10	10	10	10	10	10
L-arginine[c]	30	30	30	30	30	30
Vitamin mixture[e]	30	30	30	30	30	30
Mineral mixture[f]	60	60	60	60	60	60
α-Starch	50	50	50	50	50	50
Sucrose[c]	50	50	50	50	50	50
Glucose[c]	50	50	50	50	50	50
Glucosamine-HCl[c]	8	8	8	8	8	8
Sodium citrate[c]	3	3	3	3	3	3
Sodium succinate[c]	3	3	3	3	3	3
k-Carrageenan[g]	25	25	25	25	25	25
CMC	30	30	30	30	30	30
Ax[h]	0	2.5	5	10	15	20
α-Cellulose	61	58.5	56	51	46	41
Total	1000	1000	1000	1000	1000	1000

[a] Wako Pure Chemical Industries, Ltd. Osaka, Japan.

[b] Riken Vitamin, Tokyo, Japan.

[c] Nacalai Tesque, Kyoto, Japan.

[d] PUFA: (eicosapentaenoic acid) EPA 4 g and (docosahexenoic acid) DHA 4 g.

[e] Vitamin mix (30 g kg^{-1} diet) (Vitamin A free): *p*-aminobenzoic acid 0.23 g, biotin 0.01 g, inositol 9.17 g, nicotinic acid 0.92 g, Ca-pantothenate 1.38 g, pyridoxine-HCl 0.28 g, riboflavin 0.18 g, thiamine-HCl 0.09 g, menadione 0.09 g, α-tocopherol 0.46 g, cyanocobalamine 0.002 g, calciferol 0.03 g, stay-C 3.4 g, folic acid 0.02 g, choline choride 13.75 g.

[f] Mineral mix (60 g kg^{-1} diet): K_2HPO_4 13.98 g, $Ca_3(PO_4)_2$ 19.11 g, $MaSO_4.7H_2O$ 21.36 g, $NaH_2PO_4.2H_2O$ 5.55 g.

[g] Sigma-Aldrich, St. Louis, MO, USA.

[h] Ax: Carophyll Pink® containing 8% astaxanthin made by DSM Nutrition, Japan.

Table 2-2. Biochemical analysis of the diet (% wet weight).[1]

Parameter	Astaxanthin supplementation level (mg kg^{-1} diet)					
	0	200	400	800	1200	1600
Moisture	11.0±0.25	10.0±0.64	10.1±0.23	11.1±0.02	10.1±0.64	10.2±0.07
Crude protein	54.4±2.75	55.6±9.5	54.4±1.01	55.5±4.17	55.6±2.41	55.2±6.76
Crude lipid	8.4±0.15	8.3±0.39	8.66±0.32	8.21±0.11	8.27±0.08	8.27±0.3
Ash	5.82±0.25	5.57±0.64	5.99±0.23	5.85±0.02	5.73±0.64	5.89±0.07

[1] Data are expressed as mean ± S.E.M. from triplicate groups.

Table 2-3. Effects of dietary astaxanthin on juvenile kuruma shrimp, *Marsupenaeus japonicus*, after the 56-day feeding trial.[1]

Parameter[2]	Astaxanthin supplementation level (mg kg^{-1} diet)					
	0	200	400	800	1200	1600
SR (%)	60.0±3.85^{a}	62.2±5.88^{a}	62.2±11.76^{a}	64.4±8.01^{a}	60.0±7.70^{a}	62.2±5.88^{a}
IBW (g)	1.42±0.03^{a}	1.41±0.03^{a}	1.41±0.01^{a}	1.40±0.03^{a}	1.41±0.03^{a}	1.41±0.03^{a}
FBW (g)	2.02±0.05^{a}	2.07±0.05^{ab}	2.19±0.08^{c}	2.14±0.06^{bc}	2.11±0.04^{abc}	2.11±0.05^{abc}
BWG (%)	42.9±1.85^{a}	46.9±1.78^{ab}	55.2±3.33^{c}	52.5±2.68^{bc}	49.4±1.44^{abc}	50.0±2.07^{abc}
SGR (%)	0.59±0.02^{a}	0.64±0.02^{ab}	0.73±0.04^{c}	0.70±0.03^{bc}	0.67±0.02^{abc}	0.68±0.02^{abc}
FE	2.30±0.14^{a}	2.81±0.43^{a}	2.72±0.42^{a}	2.41±0.35^{a}	2.49±0.52^{a}	2.54±0.28^{a}

[1] Data are expressed as mean ± S.E.M. from triplicate groups. Different superscript letters indicate significant differences ($P < 0.05$).

[2] Abbreviations are as follows: survival (SR, %) = (final number of shrimp/initial number shrimp) × 100; initial body weight (IBW); final body weight (FBW), body weight gain (BWG, %) = [(final weight-initial weight)/initial weight] × 100; specific growth rate (SGR, %/day) = [(ln final weight-ln initial weight)/duration] × 100; feed efficiency (FE).

Table 2-4. Whole-body compositions (% dry weight) of juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. [1]

Parameter	Astaxanthin supplementation level (mg kg^{-1} diet)					
	0	200	400	800	1200	1600
Moisture	25.1±0.66	24.7±0.58	25.8±0.24	23.2±0.68	25.4±0.57	25.9±0.61
Crude protein	62.8±0.78	63.5±0.49	62.5±0.22	62.5±0.38	62.8±0.62	64.5±0.74
Crude lipid	6.42±0.19	6.78±0.21	6.44±0.24	6.25±0.20	6.36±0.43	6.92±0.37
Ash	18.2±0.14	18.9±0.05	20.3±0.68	19.8±0.57	19.6±0.16	20.2±0.24

[1] Data were expressed as mean ± S.E.M. from triplicate groups.

Table 2-5 Fatty acid composition of the whole bodies of shrimp separated by neutral and polar lipids.[1]

Parameter		Astaxanthin supplementation level ((mg kg^{-1} diet)					
		0	200	400	800	1200	1600
NL	SFA	22.9±0.91^{a}	30.3±0.65^{b}	31.7±0.29^{b}	24.2±0.61^{a}	22.6±0.17^{a}	30.0±0.43^{b}
	MUFA	45.6±0.69^{c}	40.8±0.77^{a}	40.6±0.13^{a}	42.6±0.05^{b}	44.1±0.24^{bc}	39.3±0.32^{a}
	PUFA	31.6±0.59^{bc}	28.9±0.69^{ab}	27.7±0.42^{a}	33.2±0.56^{c}	33.3±0.41^{c}	30.7±0.75^{abc}
	EPA	7.22±0.05^{a}	8.12±0.02^{b}	9.24±0.11^{c}	10.8±0.15^{d}	9.7±0.06^{c}	9.89±0.04^{c}
	DHA	5.58±0.07^{a}	5.53±0.17^{a}	5.05±0.34^{a}	8.12±0.21^{c}	7.37±0.33^{bc}	6.18±0.23^{ab}
PL	SFA	27.6±0.26^{ab}	24.7±0.39^{a}	25.9±0.6^{a}	31.5±0.08^{b}	26.8±0.14^{ab}	27.2±0.16^{ab}
	MUFA	35.6±0.27^{a}	35.3±0.16^{a}	33.8±0.26^{a}	32.5±0.42^{a}	32.8±0.91^{a}	32.2±0.48^{a}
	PUFA	36.8±0.96^{a}	40.0±0.24^{b}	40.3±1.45^{b}	35.9±0.49^{a}	40.5±1.23^{b}	40.6±0.32^{b}
	EPA	10.9±0.11^{a}	11.8±0.22^{abc}	13.1±0.56^{d}	11.5±0.05^{ab}	12.0±0.21^{bc}	12.5±0.03^{cd}
	DHA	9.43±0.36^{a}	10.6±0.16^{a}	10.9±0.75^{a}	10.3±0.28^{a}	10.3±0.82^{a}	11.1±0.11^{a}

[1] Data are expressed as mean ± S.E.M. from triplicate groups. Different superscript letters indicate significant differences ($P < 0.05$).

Table 2-6 Effects of dietary astaxanthin on juvenile kuruma shrimp, *Marsupenaeus japonicus*, after the 56-day feeding trial.[1]

Parameter	Astaxanthin supplementation level (mg kg^{-1})					
	0	200	400	800	1200	1600
Diet	18.3±0.18	186.5±0.09	396.7±0.29	783.7±0.61	1142.6±0.35	1540±0.43
Whole body	60.5±0.26	69.5±0.14	92.0±0.12	157.6±0.47	220.3±0.16	244.4±0.62

[1] Data were expressed as mean ± S.E.M. from triplicate groups.

Table 2-7 Color parameters of cooked whole shrimp bodies read by a colorimeter.[1]

Parameter		Astaxanthin supplementation level (mg kg^{-1} diet)					
		0	200	400	800	1200	1600
Head	L^*	61.2 ± 0.29^{c}	58.7 ± 1.24^{b}	57.8 ± 1.01^{b}	54.3 ± 0.26^{a}	58.1 ± 0.75^{b}	54.6 ± 0.56^{a}
	a^*	10.9 ± 0.82^{a}	11.7 ± 1.68^{a}	15.3 ± 0.83^{b}	16.3 ± 0.59^{b}	18.2 ± 1.64^{b}	18.5 ± 0.69^{b}
	b^*	15.7 ± 2.13^{a}	18.1 ± 0.60^{ab}	22.3 ± 1.29^{bc}	21.3 ± 1.23^{bc}	21.6 ± 0.63^{bc}	23.4 ± 1.65^{c}
Body	L^*	60.8 ± 0.95^{c}	60.8 ± 0.63^{c}	59.5 ± 0.23^{bc}	58.7 ± 0.91^{abc}	56.7 ± 0.58^{a}	58.3 ± 0.16^{ab}
	a^*	10.8 ± 1.44^{a}	13.3 ± 0.87^{ab}	14.5 ± 0.31^{ab}	15.5 ± 0.42^{b}	15.4 ± 0.87^{b}	15.9 ± 0.72^{b}
	b^*	16.8 ± 1.10^{a}	20.1 ± 0.42^{ab}	19.3 ± 1.61^{ab}	20.3 ± 1.11^{ab}	21.1 ± 1.08^{b}	20.7 ± 0.82^{b}

[1] Data are expressed as mean ± S.E.M. from triplicate groups. Different superscript letters indicate significant differences ($P < 0.05$).

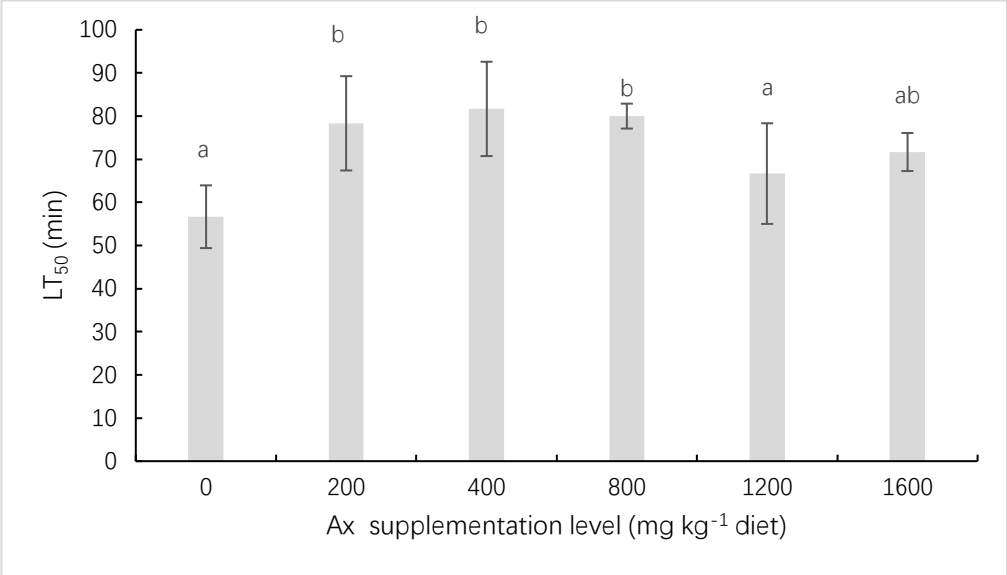

Fig. 2-1. Time to 50 % mortality (min) after the fresh water stress test of juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with the experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups. Means with different lower-case letters are significantly different ($P < 0.05$).

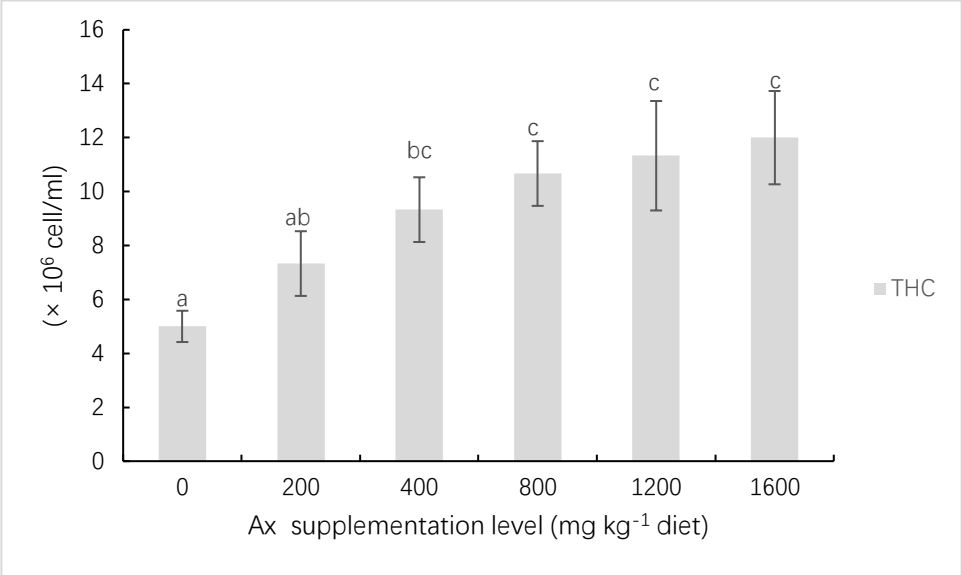

Fig. 2-2. Total haemocyte count of juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups. Means with different lower-case letters are significantly different ($P < 0.05$).

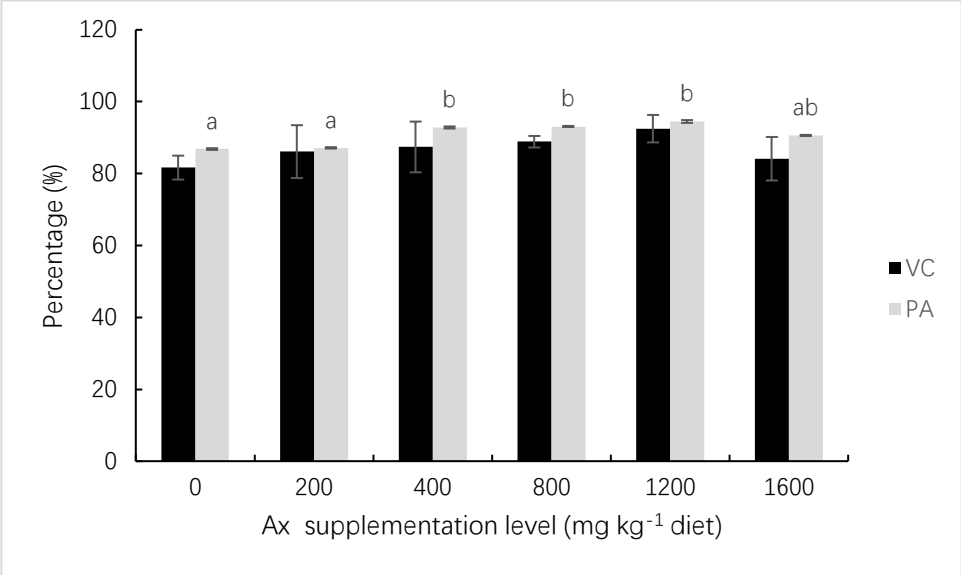

Fig. 2-3. Viable cell (%) and phagocyte activity of juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups. Means with different lower-case letters are significantly different ($P < 0.05$).

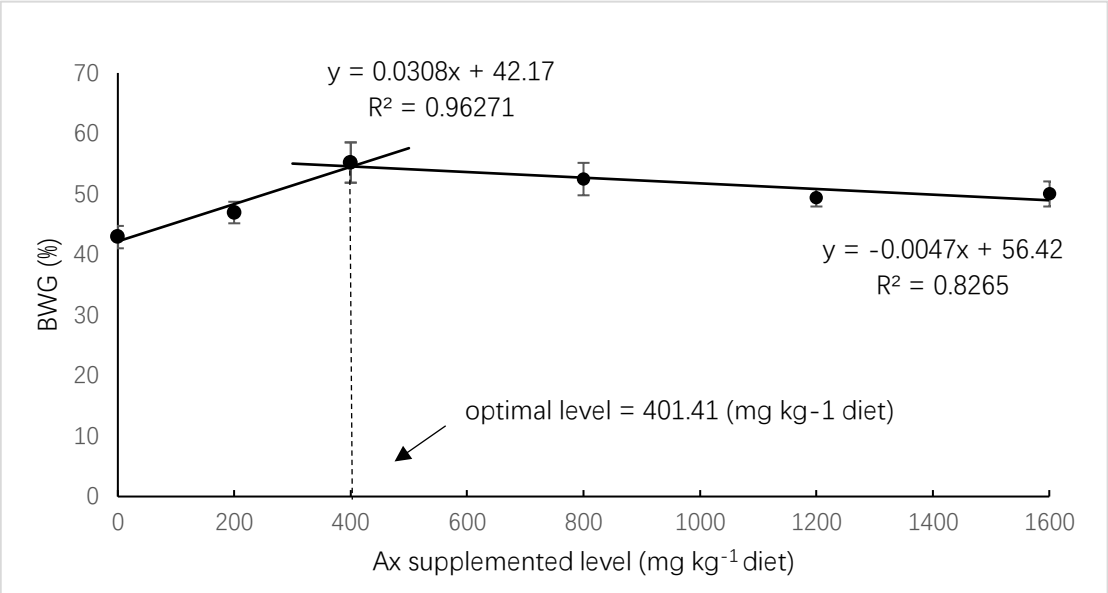

Fig. 2-4. Broken-line analysis of body weight gain on juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups.

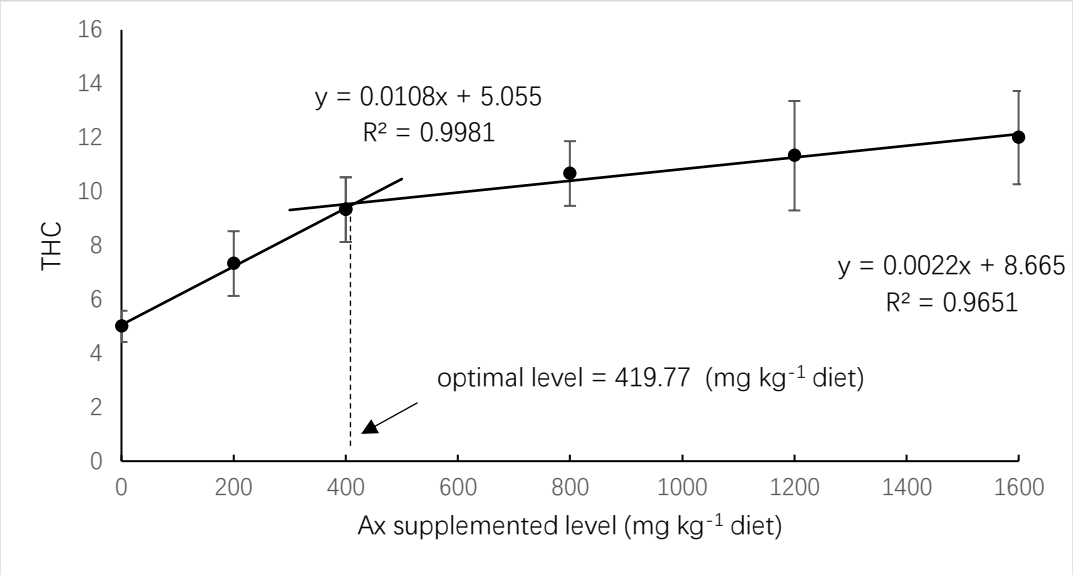

Fig. 2-5. Broken-line analysis of total hemocyte count on juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups.

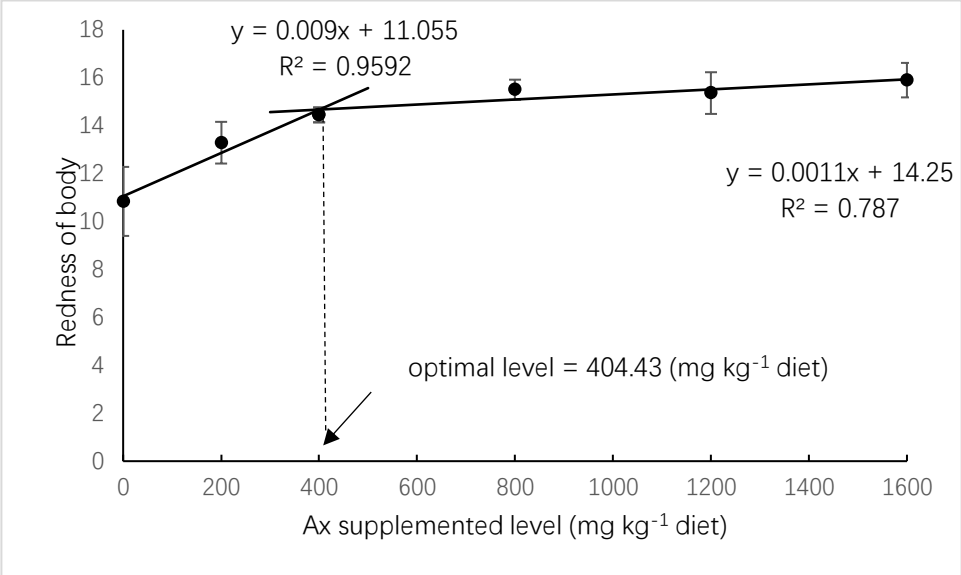

Fig. 2-6. Broken-line analysis of shrimp body redness (*a**) in juvenile kuruma shrimp, *Marsupenaeus japonicus*, fed with experimental diets for 56 days. Values are expressed as mean ± S.E.M. from triplicate groups.

Chapter 4: Interactive Effects of Various Dietary Factors on Astaxanthin Absorption in Kuruma Shrimp

***Study 3*: Interactive Effects of Dietary Astaxanthin and Vitamin E on the Kuruma Shrimp (*Marsupenaeus japonicus)* Performance**

Abstract

A 56-day feeding trial was done to investigate the interactive effects of astaxanthin (Ax) and vitamin E (α-Toc) on the performance of juvenile kuruma shrimp (*Marsupenaeus japonicus*). A 2 × 3 factorial experiment was conducted with six experimental diets containing two levels of Ax (0 and 0.6 g kg^{-1} diet (Ax_0 and $Ax_{0.6}$, respectively)) and three levels of α-Toc (0, 0.2, and 1 g kg^{-1} diet (α-Toc_0, α-$Toc_{0.2}$, and α-Toc_1, respectively)). The results indicated that growth performance was highest in shrimp fed with the $Ax_{0.6}$ × α-$Toc_{0.2}$ diet. Interactive effects between Ax and α-Toc on the growth parameters were observed. Further, pigmentation performance was significantly better in the $Ax_{0.6}$ groups. Interaction between Ax and α-Toc was also found in the Ax content of shrimp body parts. Interestingly, dietary α-Toc may help to reduce the Ax consumption rate, promote the absorption, and increase deposition of Ax in the muscle. Kuruma shrimps from the $Ax_{0.6}$ groups showed improved hepatopancreatic digestive enzyme activities compared with those of the Ax_0 groups. Although no interactive effects were found between dietary α-Toc and Ax on total hemocyte count and tolerance against freshwater, dietary Ax and α-Toc supplementation showed better performance on these two parameters. It was concluded that dietary Ax and α-Toc functioned interactively, and the shrimp fed with the diet containing 0.6 g Ax kg^{-1} diet Ax and 0.2 g α-Toc kg^{-1} diet showed improved growth and pigmentation performance compared with the other groups in the current study.

Keywords: Astaxanthin; Vitamin E; Interaction; Growth; Coloration; Immune response; Kuruma shrimp

Introduction

Carotenoid pigments are widely used for flesh pigmentation of aquatic animals and constitute a major part of the feed cost (Chimsung et al., 2014). Carotenoid pigments cannot be synthesized by aquatic animals themselves; therefore, they need to be supplemented in the diet (Meyers and Latscha, 1997). Astaxanthin (Ax) is one carotenoid that is commonly used for primary pigment in crustaceans, with the greatest proportion found in the exoskeleton (Katayama et al., 1972). Petit et al. (1998) also found that Ax is more efficiently assimilated than other carotenoids. D' Abramo et al. (1983) demonstrated that the concentration of coloration in juvenile lobsters is directly related to dietary supplementation with different carotenoid compounds that compound to Ax as the end-product of biosynthetic pathways.

For shrimp, coloration depends on the presence of Ax within hypodermal tissues (Boonyaratpalin et al., 2001). Wade et al. (2012) also showed that the color change in shrimp was associated with hormonally controlled expansion and contraction of chromatophores within the hypodermal layer. However, the biochemical mechanisms involved in the pathway of carotenoids' deposition are not clear and have been subject to limited investigation.

Cultured kuruma shrimp that have a light-pink after cooking, rather than a deep-red, color like wild populations, are considered to have an insufficient level of carotenoids in their diet due to high-density cultivation. Therefore, it is necessary to provide Ax to improve pigmentation and enhance commercial value.

Ax plays a vital role not only in pigmentation, but also in growth performance (Niu et al., 2012), as the precursor of vitamin A (Miki et al., 1982) and as an antioxidant (Linan-Cabello et al., 2002), and the overall health of marine animals. However, the effects of dietary Ax on the physiological performance of shrimp are varied, and the efficiency of Ax uptake from the diet and deposition in shrimp tissues is poorly defined (Wade et al., 2017). Several factors such as shrimp size, developmental stage (post-larval, juvenile, and adult), methodological differences, pigment source, losses during feed processing and storage, diet composition, and genetic differences can affect the utilization and retention of Ax (Metusalach et al., 1996; Wade et al., 2017).

Certain dietary factors such as vitamin E (α-Toc), cholesterol, fiber, lutein, zeaxanthin, and phytosterol are known to influence absorption of Ax in animals (Yeum and Russel, 2002). Chimsung et al. (2014) showed that these ingredients are likely to affect intestinal absorption

of Ax and metabolism in salmon. An improvement in Ax deposition and coloration of Atlantic salmon was found by increasing dietary *α*-Toc levels (Bjerkeng et al., 1999). *α*-Toc protects critical cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation (Lee and Shiau, 2004). Previous investigations have implied that a direct or indirection interaction may exist between α-Toc and Ax in shrimp.

However, no data were available regarding the interactive effect between dietary *α*-Toc and Ax supplementation in kuruma shrimp. Our study focused on the interactive effects of dietary *α*-Toc and Ax supplementation on growth performance, immune parameters, stress test, color reading, and digestive enzyme activities in kuruma shrimp.

Materials and Methods

Astaxanthin Preparation

I used a chemically synthesized pigment (Carophyll® Pink, DSM Nutrition, Japan), containing 10% Ax, for our experiments.

Test Shrimp and Experimental System

Juvenile kuruma shrimp (*Marsupenaeus japonicus)* were obtained from Matsumoto, Miyazaki, Japan, and transported to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University. The juveniles were maintained in two 500 L black polyethylene circular tanks without a sandy bottom at an ambient water temperature of 23 ± 0.5 °C and fed with commercial diet (Higashimaru Feeds Ltd., Kagoshima, Japan) for one week to acclimatize. I used 18 black rectangular polyvinyl chloride (PVC) tanks (32 L) during the feeding trial. The tanks were equipped with a continuous aeration and water circulating system by filtration through a sand filter covered with a net. Water was circulated at 0.6 L min^{-1} during the experimental period. Each tank was covered with black plastic film and a net to reduce algal growth as a potential external source of carotenoids, minimize disturbance, and prevent

shrimp from jumping out. Ten juveniles (average size = 2.5 ± 0.15 g initial wet weight) were randomly selected from the stock tanks and transferred to the experimental tanks, for a total of three tanks for each of the six diets (Table 1). Sand (about 20 mm thickness) was distributed on the bottom of each tank.

Diet Preparation

Tables 3-1 and 3-2 summarize the composition and chemical analysis of the experimental diets. Based on nutritional composition, six diets were formulated to be isonitrogenous (46% crude protein) and isolipidic (8% lipid), with varying Ax (0 and 0.6 g kg^{-1} diet (Ax_0 and $Ax_{0.6}$, respectively)) and α-Toc levels (0, 0.2, and 1 g kg^{-1} diet (α-Toc_0, α-$Toc_{0.2}$, and α-Toc_1, respectively)). Each diet was initially mixed with the dry ingredients in a food mixer for 10 min. Pollack liver oil, soybean lecithin, and fat-soluble components were premixed with a sonicator (CA - 4488Z, Kaijo Corporation, Tokyo, Japan), then added to the dry ingredients and mixed for another 10 min. Water was added gradually (35-40% of the dry ingredients) to the premixed ingredients and mixed for another 10 min. The pH of the mixtures (dough) were adjusted 7.0-7.5 with 1 N sodium hydroxide. The dough was then passed through a screw-pressed single-screwed mincer (ROYAL Inc., Tokyo, Japan) to prepare pellets (1.2-2.2 mm). Pellets were then dried in a convection oven (DK400, Yamato Scientific, Tokyo, Japan) at 60 °C until moisture content decreased to 10%. The dried pellets were steamed at 100 °C for 1 min in a cylindrical steamer to improve water stability and stored at -20 °C until use.

Feeding Protocol

Triplicate groups of juveniles were fed twice daily for 56 days with the test diets at a ration equal to 8-10% of body weight. Daily proportions were divided into 20% at 0800 h and 80% at 1700 h. I recorded uneaten pellets using a well-established method that converts pellet counts to gram weight, based on average pellet weight (Wade et al., 2017). I removed the uneaten diet from the tanks, together with fecal matters, using a siphon every morning. Every 14 days,

juveniles were bulk-weighted and the survivors in each tank were counted. Meanwhile, the tank and sand were cleaned and filled with fresh water.

Sample Collection and Biochemical Analysis

At the beginning of the experiment, 15 juveniles from the stock were sampled for analysis of whole-body composition and stored at -20 ℃. At the end of the experiment, all shrimp were fasted for 24 h prior to final sampling. The total number of survivors and individual body weight of shrimp from each tank were measured. Proximate analysis of the diets and whole-body shrimp were analyzed by standard AOAC methods (AOAC, 1990), including moisture by oven-drying at 110 ℃ to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method, and ash by combustion in a muffle furnace at 550 ℃ for 4 h. The total hemocyte count (THC) method described by Itami et al. (1998) was applied with slight modification.

Determination of Vitamin E Content in the Diet

The freeze-dried sample was homogenized with methanol, chloroform, water (2:2:1.8 v/v/v) for extracting the total lipid per Bligh and Dyer (1959). Then, the total lipid was dried with a stream of nitrogen at 40 ℃. I dissolved the residue in 0.8 mL 50% KOH and 2 mL 1% pyrogallol in 60% methyl alcohol per Nguyen et al. (2012) with slight modification. The saponification reaction lasted for 50 min in 85 ℃ water bath. After cooling, 1 mL distilled water and 2.4 mL hexane were added, and the hexane layer was removed. I filtered the hexane layer through a 0.45 μm cellulose acetate membrane filter (Advantec, Toyo Roshi Co., Japan) and evaporated the hexane in a rotary vacuum evaporator (Eyela SB-1100 and CCA-1111, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The residue was dissolved in 1 mL isopropanol and stored in brown vials. α-Toc was analyzed by high-performance liquid chromatography (HPLC), using a Cosmosil 5C_{18}-MS4.6 × 150 mm column and SHIMADZU RF-535 fluorescence detector at (EX = 298 nm, EM = 325 nm) under the operational conditions: mobile

phase, isopropyl alcohol/acetonitrile/water = 67.5/25/7.5 (v/v/v); solvent flow rate, 0.7 mL min^{-1}; and injection volume, 30 µL. The standard used DL-*α*-tocopherol (Wako Pure Chem. Ind. Ltd., Japan). The standard curve was presented by the equation Y = 0.0032x + 0.146 (R^2 = 0.99).

Color Measurement

The bags of samples (six shrimp per bag) were put into a boiling water bath for 3 min. The bags were then cooled in tap water (about 5 ℃) in darkness per Ju et al. (2011), with slight modifications. Ando et al. (2014) stated that kuruma shrimp cooked at 100 ℃ and stored at 5 ℃ showed better color development rate. Whole-body shrimp photos were taken using a digital camera with 24 mm focal length under natural sunlight. For color assessment, we used a colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Inc., Osaka, Japan) to identify color differences according to CIE $L^*a^*b^*$ coordinates (Nickell and Bromage, 1998): L^*, lightness; a^*, redness; and b^*, yellowness.

Astaxanthin Analysis of Whole-body Shrimp

The shrimp samples were dissected into shell (including carapace, telson, and uropod), flesh, and head (including visceral). The dissected parts were weighted and freeze-dried, then crushed into powder and placed into a 50 mL polypropylene centrifuge tube for Ax analysis per Masako et al. (2010), with slight modifications. Then, the samples were extracted with chloroform by ultrasonic mixing for 10 min and centrifuged at 4 ℃ at 11,800 rpm for 15 min. I transferred the extracted Ax to flasks, then dried them in a rotary vacuum evaporator (Eyela SB-1100 and CCA-1111, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). I dissolved the sample extracts in 10 mL acetone solution. The enzyme reaction lasted for 120 min at 37 ℃ by adding 6 mL 0.05 M Tris-HCl buffer (pH 7.0) and 600 µL cholesterol esterase solution (60 units mL^{-1}). After the enzyme reaction, the solution was transferred to a 250 mL separatory funnel, partitioned with 10 mL petroleum ether, and washed three times with 0.01 g $NaSO_4$ to remove residual acetone. Then, using the rotary vacuum evaporator, the solvent was mobilized and filtered through a 0.2

μm Millipore filter and stored in brown vials. Ax was analyzed by high-performance liquid chromatography (HPLC), using a shim-pack VP-ODS (150mmL. × 4.6mmL.D.) and SPD-M20A detector at 475 nm under the operational conditions: mobile phase, 0.05% Trifluoracetic Acid/Methanol = 3/97 (v/v); solvent flow rate, 1.2 mL min^{-1}; and injection volume, 10 μL. The standard used Carophyll® Pink (10% Ax). The standard curve was presented by the equation $Y = 60595x - 9081$ ($R^2 = 0.99$).

Digestive Enzyme Activities

Protease activity was analyzed using hepatopancreas of shrimp from three tanks for each diet group using Sigma's Non-specific Protease Activity Assay (Cupp-Enyard, 2008), with slight modifications. The enzyme assays used casein as the substrate and Folin-phenol as the reagent. The aliquots of diluted enzymatic extract for each sample were incubated at 37 ℃ for 10 min after adding 5 mL of 0.65% casein. I stopped the reaction by adding 5 mL 110 mM trichloroacetic acid (TCA) and incubating at 37 ℃ for 30 min. I filtered the mixture using a 0.45 μm syringe; then, I added 5 mL 500 mM sodium carbonate solution and 1 mL of 0.5 mM Folin's reagent and incubated it at 37 ℃ for 30 min. After incubating, using the 0.45 μm syringe filter to filter again for better results, I measured the intensity of light at 660 nm with the spectrophotometer. The protease activity was determined from the 1.1 mM tyrosine standard curve (0, 0.05, 0.1, 0.2, 0.4, and 0.5 mL) and I obtained enzyme in units mL^{-1}:

Units mL^{-1} Enzyme = [μmole tyrosine equivalents released × (11)]/[(1) × (10) × (2)]

Where 11 = total volume (mL) of assay, 10 = time of assay (min), 1 = volume of enzyme used (mL), and 2 = volume (mL) used in colorimetric determination.

I analyzed amylase activity using starch as the substrate per Shiu et al. (2015), with slight modifications. I added 0.5 mL enzyme solution and incubated the tubes at 25 ℃ for 3-4 min to achieve temperature equilibration. At timed intervals, I added 0.5 mL starch solution and incubated the tubes for exactly 3 min at 25 ℃. At timed intervals, I added 1mL dinitrosalicylic acid color reagent to each tube and incubated them for 5 min in a boiling water bath. Then,

they were cooled to room temperature and I added 10 mL distilled water to each tube. The tubes were mixed well and the absorbance of light with a wavelength of 540 nm was read by a U-1900 HATCH spectrophotometer (Hitachi High-Technologies Corportaion, Japan) and compared to a blank. I determined the μmoles of maltose released from a standard curve made by maltose stock solution.

Lipase activity was analyzed per Mustafa et al. (2016), with slight modifications. I prepared olive oil emulsion by mixing 50 mL of olive oil with 150 mL 4% polyvinyl alcohol solution in a homogenizer. The reaction mixture was incubated at 40°C for 15 min containing 2 mL of olive oil emulsion, 2.5 mL of 20 mM phosphate citrate buffer (pH 7.5), and 0.5 mL enzyme solution. Then, I stopped the reaction immediately after incubation, by adding 1 mL 6 M HCl and 6 mL 95% ethanol. After mixing well, I collected 1 mL of the supernatant fluid, added 4 mL of isooctane and 1 mL 5% cupric acetate pyridine reagent (pH 6.1) for reading by the spectrophotometer at A_{714} versus blank.

Stress Resistance Test

Freshwater osmotic tests were conducted after the feeding trial. Shrimp were challenged with low-salinity conditions to investigate the tolerance against stressors and evaluate the efficiency of test diets. Five homogenously sized test shrimp from each experimental tank were put in 10 L glass bottles. The bottles were filled with 8 L tap water that was dechlorinated by aeration for 24 h before starting the stress test. Mortality was monitored every 20 min. Survival was expressed as the LT_{50}: the time required for 50% mortality of test shrimp by stressors. I followed the calculation per Hossain et al. (2016): the time to death (min) was recorded and the survival values were converted to $\log_{10}$ (survival). These values of $\log_{10}$ (survival) were plotted against the time to the death to determine the time until 50% mortality of shrimp in each treatment. Each value obtained from the equation was compared statistically. The higher the value, the greater the tolerance stressors:

$$Y = aX + b$$

Where Y = $\log_{10}$ (survival) and X = time to individual death. LT_{50} (X) was obtained when Y = 1.7, since $\log_{10}$ (50) = 1.7

Statistical Analysis

The statistical analysis was performed using analysis of variance (ANOVA) (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). All data were presented as mean values standard ± error of mean (S.E.M., n = 3), Data from each group were compared using Tukey-Kramer. Differences between treatments were considered significantly when $P < 0.05$. Two-way ANOVA was employed to test the effects of dietary α-Toc levels and Ax levels, as well as their interactions.

Results

Growth Performances and Survival

Growth performance, survival, and intermolt period (IP) of the test shrimp fed with different diets for 56 days are provided in Table 3-3. Dietary α-Toc and Ax supplementations and the interaction between these two additives did not significantly affect survival ($P > 0.05$). However, dietary α-Toc and the interaction between α-Toc and Ax significantly affected final body weight (FBW), body weight gain (BWG), and specific growth rate (SGR) ($P < 0.05$). Only α-Toc significantly affected feed conversion ratio (FCR) and feed intake (FI). The interaction between dietary α-Toc and Ax was the only factor that significantly affected IP.

The fastest growth was found in shrimp fed with a high level of Ax and an intermediate level of α-Toc ($Ax_{0.6}$ × α-$Toc_{0.2}$ group), and the mean values were significantly higher than the control and $Ax_{0.6}$ × α-Toc_1 groups. FBW, BWG, and SGR in the Ax_0 groups showed an increasing trend with increased α-Toc supplementation, and the Ax_0 × α-Toc_1 group showed significantly higher FBW than the control group ($P < 0.05$). The IP was shorter in the $Ax_{0.6}$ groups than in the Ax_0 groups. However, there were no differences among all the treatments.

Whole-body Proximate Analysis

The whole-body proximate analysis of kuruma shrimp fed with experimental diets for 56 days is shown in Table 3-4. Dietary α-Toc and Ax supplementations and the interaction between these two additives did not significantly affect moisture, crude protein, or ash content ($P > 0.05$). However, α-Toc significant affected crude lipid ($P = 0.01$).

Shrimp Pigmentation

The color parameters for cooked shrimp are shown in Table 3-5. The interaction between dietary Ax and α-Toc were significantly affected L^* of the head, b^* of the shell, and a^* of shrimp muscle. Dietary Ax was a significant factor for all groups ($P < 0.05$) expect yellowness (b^*) of the muscle ($P = 0.36$).

L^* of head, shell, and muscle was greater in the Ax_0 groups than in the $Ax_{0.6}$ groups. For the head, the Ax_0 groups showed an increasing trend with increased α-Toc supplementation. A similar increasing trend was also found in the Ax_0 groups for the L^* of the shell and muscle parts. a^* of muscle in the $Ax_{0.6}$ groups was significantly higher than in the Ax_0 groups. However, no significant difference was found among the Ax_0 or $Ax_{0.6}$ groups ($P > 0.05$), respectively. For a^* of the muscle, the increasing trend with the increased α-Toc supplementation was lower in both Ax_0 and $Ax_{0.6}$ groups. However, the $Ax_{0.6} \times \alpha\text{-Toc}_1$ group showed the highest a^* values of head, shell, and muscle. b^* of carapace showed an increasing trend with increased α-Toc supplementation in the $Ax_{0.6}$ groups. Moreover, the $Ax_{0.6} \times \alpha\text{-Toc}_{0.2}$ and $Ax_{0.6} \times \alpha\text{-Toc}_1$ groups exhibited significantly higher values than the control group.

Total Astaxanthin Content in Different Shrimp Body Parts

Total Ax content (free Ax + esterified Ax) in whole body, shell, muscle, and head of kuruma shrimp fed with experimental diets for 56 days is shown in Table 3-6. Dietary α-Toc and Ax supplementation and the interaction between the two additives significantly affected Ax

content in different shrimp body parts ($P < 0.05$). However, dietary α-Toc and Ax supplementation and the interaction between the two additives did not significantly affect the yield (%) of different body components ($P > 0.05$). Compared to the Ax_0 groups, total Ax in the $Ax_{0.6}$ groups was significantly higher ($P < 0.05$). In the $Ax_{0.6}$ groups, increases in dietary α-Toc increased whole body and muscle.

Digestive Enzyme Activities

The digestive enzyme activities of kuruma shrimp fed with experimental diets for 56 days is shown in Table 3-7. Dietary α-Toc and Ax supplementation and the interaction between these two additives significantly affected lipase activity of shrimp hepatopancreas ($P < 0.05$). Dietary α-Toc and Ax supplementation significantly affected protease activity of the shrimp hepatopancreas ($P < 0.05$). Only Ax significantly affected amylase activity.

Although, the α-Toc supplemented groups in the Ax_0 groups showed higher values than the control group, there was no significant difference among the groups. Dietary Ax significantly affected the protease, lipase, and amylase activity, and $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ showed better performance than the Ax_0 groups and $Ax_{0.6} \times \alpha\text{-}Toc_0$ groups. However, there were no significant differences between the $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ and $Ax_{0.6} \times \alpha\text{-}Toc_1$ groups.

Immune Response

The result for THC as immune response parameters are shown in Figures 3-1. Although no interactive effect was found by dietary α-Toc and Ax supplementation on THC, dietary Ax and α-Toc supplementation significantly increased immune response, respectively. Higher THC was observed in the $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ and $Ax_{0.6} \times \alpha\text{-}Toc_1$ groups. The $Ax_0 \times \alpha\text{-}Toc_{0.2}$, $Ax_0 \times \alpha\text{-}Toc_1$, and $Ax_{0.6} \times \alpha\text{-}Toc_0$ groups showed intermediate values. The lowest THC was given by the control group ($Ax_0 \times \alpha\text{-}Toc_0$).

Fresh Water Stress Test

The results of the lethal stress test against fresh water shock on LT_{50} (h), obtained by regression analysis, are shown in Figure 3-2. Although no interactive effect was found by dietary α-Toc and Ax supplementation on LT_{50} of kuruma shrimp, supplementation significantly increased tolerance against freshwater stress. The $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ and $Ax_{0.6} \times \alpha\text{-}Toc_{1}$ groups showed significantly better performance than the control and $Ax_{0} \times \alpha\text{-}Toc_{0.2}$ groups.

Discussion

Carotenoids are widely distributed pigments in the animal kingdom, particularly in crustaceans (Petit et al., 1998). Farmed shrimp typically have a different shade of redness compared to wild shrimp; therefore, crustaceans are reported to use de novo synthesis of carotenoids, with wild shrimp consuming a varied diet of algae, fish, and crustaceans that contain carotenoids (Sigurgisladottir et al., 1994). Many studies have shown that Ax is the most important natural red pigment found in salmon, lobster, crab, shrimp, and red snapper (Sigurgisladottir et al., 1994; Torrissen et al., 1990). And many have also shown that dietary supplementation with Ax produces better pigmentation performance in shrimp, compared to pure carotenoids or crude carotenoids (Chien et al., 1992; Yamada et al., 1990). Previous studies have investigated the optimal levels of Ax for pigmentation performance by dietary supplementation with synthetic Ax (Carophyll® Pink). Yamada et al. (1990) found that pigmentation of kuruma shrimp increased to the highest level with 0.2 g Ax kg^{-1} diet, and Chien et al. (1992) showed that the most effective level of Ax for optimal pigmentation in kuruma shrimp was 1 g kg^{-1} diet. The effects of dietary Ax in previous studies are varied. Several factors such as shrimp size, diet composition, and environmental conditions may affect utilization and retention of Ax. The interaction among ingredients can also affect the efficiency of Ax uptake from the diet and deposition in shrimp tissues. Although the metabolic relationship is poorly understood, the results of our research indicate several statistically significant interactions between α-Toc and Ax.

After a 56-days feeding trial, the survival rate of shrimp fed with different levels α-Toc and Ax did not show significant differences ($P > 0.05$) among treatments. This result is consistent with previous findings (He and Lawrence, 1993; Ju et al., 2011; Wade et al., 2017), which also found that dietary different levels Ax and α-Toc supplementation did not significantly affect shrimp survival rate. The highest growth performances, in terms of FBW, BWG, and SGR, were found in the $Ax_{0.6}$ × α-$Toc_{0.2}$ group, indicating that kuruma shrimp fed with this diet showed a higher growth performance than the other groups in our study. Two-way ANOVA revealed that dietary Ax did not significantly affect growth performances, which agrees with previous studies (Boonyaratpalin et al., 2001; Chien and Jeng, 1992). Although BWG and SGR increased with the increasing α-Toc in the Ax_0 groups, there were no significant differences among the groups. This concurred with He and Lawrence (1993), who reported that *Penaeus vannamei* fed diets containing 0 to 0.1 g Ax kg^{-1} diet exhibited significantly increased weight gain, but no significant difference was observed for shrimp fed diets containing α-Toc from 0.1 to 0.6 g kg^{-1} diet. Physiological research can partly explain the limited ability for growth stimulation by α-Toc supplementation in the present study. On the other hand, in the $Ax_{0.6}$ groups, although FBW, BWG, and SGR increased when α-Toc was supplied at an intermediated level ($Ax_{0.6}$ × α-$Toc_{0.2}$), these parameters were significantly lower in the $Ax_{0.6}$ × α-Toc_1 group, compared with the $Ax_{0.6}$ × α-$Toc_{0.2}$ group. In the case of $Ax_{0.6}$ × α-Toc_1 group performance, the interaction between α-Toc and Ax was significant. Previous studies (Lee and Shiau, 2004) reported that a high level α-Toc depressed the growth parameters of *Penaeus monodon*, which is consistent with the $Ax_{0.6}$ groups in the present study. Although there is no significant difference in FCR among treatments, FCR decreased with increasing α-Toc, not only in the Ax_0 groups but also in the $Ax_{0.6}$ groups. Similar results were also reported for juvenile *Penaeus monodon* (Lee and Shiau, 2004): feed efficiency ((final body weight-initial body weight)/feed intake) reached a maximum at 75-100 mg α-Toc and decreased thereafter. The $Ax_{0.6}$ groups showed a shorter IP than the Ax_0 groups. This concurred with Flores et al. (2007), who reported that 0.08 g Ax kg^{-1} diet can shorten the molting cycle of white shrimp, *Litopenaeus vannamei,* acclimated to low-salinity water. Petit et al. (1997) also reported that 0.06 g Ax kg^{-1} diet modified molt frequency, shortened the molting cycle, and improved post-larval development of kuruma shrimp. Previous investigations implied that Ax can impact

molting physiology in shrimp by increasing hemolymph ecdysteroid, which is quickly hydroxylated in crustaceans and transformed into the active form of the molt-stimulant hormone (Petit et al., 1997).

Zhang et al. (2013) reported that dietary Ax did not induce significant differences in moisture, crude protein, crude lipid, and ash content of Pacific white shrimp. A similar result was found in our study. No data are available on shrimp whole-body proximate compositions when fed with different levels of α-Toc. In the present study, the significantly higher crude lipid contents in the Ax_0 × α-$Toc_{0.2}$ and $Ax_{0.6}$ × α-$Toc_{0.2}$ groups illustrated that α-Toc has a limited effect on lipid deposition and excess α-Toc may have a negative interaction on lipid deposition. This result is consistent with the research in Japanese seabass that showed α-Toc supplementation from 0 to 0.06 g kg^{-1} significantly increased muscle lipid and then decreased it at 0.2 g kg^{-1} (Zhang et al., 2016).

Color was successfully quantified using a colorimeter. The a^* color parameters explain the color differences seen in cooked shrimp that were supplemented with α-Toc and Ax. By visual inspection of the head, shell, and muscle of cooked shrimp, the $Ax_{0.6}$ groups clearly showed a more pronounced red color than the pink color of shrimp fed with the Ax_0 diets. The a^* values in our study were significantly higher in the $Ax_{0.6}$ groups than in the Ax_0 groups. In the Ax_0 groups, the total Ax content increased when α-Toc was supplied at an intermediate level (Ax_0 × α-$Toc_{0.2}$), the highest level of α-Toc (Ax_0 × α-Toc_1) significantly depressed the Ax content compared to the Ax_0 × α-$Toc_{0.2}$ group. This finding, combined with the pigmentation results in the Ax_0 groups, showed no significant differences in a^* between the Ax_0 groups. Wade et al. (2017) illustrated that carotenoid digestibility improved as dietary carotenoid levels increased in tiger shrimp. Their study showed that, after six weeks, whole-body carotenoid levels were significantly depleted in the treatments with lower levels of Ax. The current and previous studies speculate that an appropriate level of α-Toc can help to reduce the Ax consumption rate. Notably, the interaction between dietary Ax and *α*-Toc significantly affected Ax content in each shrimp body part. However, the increasing trend was only observed in the intermediate α-Toc group ($Ax_{0.6}$ × α-$Toc_{0.2}$), and there was no significant difference between the $Ax_{0.6}$ × α-$Toc_{0.2}$ and $Ax_{0.6}$ × α-Toc_1 groups for the shell and head. The interaction between dietary Ax and *α*-Toc on a^* was only found in shrimp muscle. Like all crustaceans, pigmentation is known

to be produced by the interaction between Ax and a protein called crustacyanin (CRCN) (Zagalsky, 1985). This interaction turns the color of Ax from red to blue; the interaction is disrupted and red color is released when the shrimp is cooked. As Wade et al. (2017) reported, crustaceans convert different carotenoids into Ax. And all crustacean species carry free and esterified forms of various carotenoids, predominantly Ax. Increased levels of dietary carotenoid resulted in the accumulation of carotenoid esters in crustaceans (Yamada et al., 1990). Considering our results, improved pigmentation expression in cooked shrimp was directly or indirectly affected by the levels of Ax deposition. Dietary α-Toc might promote absorption and increase deposition of Ax in the shell of shrimp, but it involves quantitative modifications of chromatophores for pigment concentration or composition within shrimp muscle.

Bjerkeng et al. (1999) reported that the retention of dietary carotenoids in muscle depends on intestinal absorption, metabolism, tissue uptake, and excretion in salmon. The anatomical characteristics of fish and shrimp digestive tracts vary greatly, and seem to be more complicated in shrimp than in fish. The hepatopancreas of shrimp combines the functions of the pancreas, intestine, and liver; thus, it is responsible for processes such as digestive enzymes, absorption of digested material, and metabolism of lipids, carbohydrates, and minerals. The digestive enzyme activity in crustacean plays a central role in nutritional physiology and may directly or indirectly regulate growth, molting cycle and complex dietary formulation (Lovett and Felder, 1990; Moullac et al., 1996). However, Ax's role as an essential nutrient for shrimp is poorly understood. In the present study, dietary Ax supplementation significantly improved ($P < 0.05$) protease activity in the $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ and $Ax_{0.6} \times \alpha\text{-}Toc_{1}$ groups, compared to all the Ax_{0} groups. Kuruma shrimp is believed to require more dietary protein for growth than other crustacean species (Bulbul et al., 2016). The higher level of protein enzyme activity obtained with diets containing a high level of Ax and an intermediate level of α-Toc ($Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ group), which might in turn explain the better growth performance showed by $Ax_{0.6} \times \alpha\text{-}Toc_{0.2}$ group. Dietary α-Toc, Ax supplementations and interaction between two additives were significant factors ($P < 0.05$) on lipase activity in the present study. This effect also suggests that the difference in whole body crude lipid content of shrimp fed with Ax and α-Toc supplemented diets might be due to improved nutrition catabolism and digestion ability.

Moreover, Ax are known as the lipid-soluble compounds. Therefore, the amount and type of fat present in shrimp tissues may influence Ax bioavailability (Regost et al., 2004). The better coloration performance and higher Ax content showed by $Ax_{0.6}$ groups might be due to strong elevation of lipase activity.

Total hemocyte count (THC) varies in crustaceans in response to infectious diseases, environmental factors, and the molt cycle (Bachere, 2000). My results showed that dietary α-Toc and Ax significantly affect the THC, consistent with previous studies (Flores et al., 2007; Lee and Shiau, 2004). Liu et al. (2004) reported that when shrimp were exposed to different environmental stress factors, the decrease in THC was correlated with a higher susceptibility to viral and bacterial diseases. In the current study, the increasing trend in immune response to dietary α-Toc and Ax treatments indicated that α-Toc and Ax can be used to elevate the general defense barrier of the organism and reduce the risk of disease.

The lethal stress test was used to assess health status by measuring stress tolerance in the experimental shrimp. In the Ax_0 groups, the LT_{50} for kuruma shrimp fed that were exposed to freshwater showed an increasing trend with increasing α-Toc. Previous investigations have implied that α-Toc exhibits an effective antioxidant role by regulating osmotic balance and resistance to salinity changes in Pacific white shrimp (Liu et al., 2004). A similar trend was also observed in the $Ax_{0.6}$ groups. The $Ax_{0.6}$ groups showed significantly better resistance to reduced salinity compared to the Ax_0 groups. Chew (1995) implied that Ax plays an important role in animal health as an antioxidant, through inactivation of free radicals produced from normal cellular activity and various stressors. Shimidzu et al. (1996) also implied that the antioxidant activity of Ax was approximately 10 times stronger than β-carotene and 100 times greater than α-Toc, which was consistent with our results.

Conclusion

In conclusion, dietary Ax and α-Toc functioned interactively on growth performance and Ax content in kuruma shrimp. However, no interactive effect was shown in the other parameters. Interestingly, dietary *α*-Toc may help to reduce the Ax consumption and increase deposition of Ax in the muscle. The current study fills a data gap regarding the interactive effects of these two supplements and provides practical information to improve pigmentation and performance of kuruma shrimp, thus enhancing their commercial value.

Table 3-1 Composition of the experimental diets (g kg^{-1}, dry matter basis).

Ingredients	Ax_0			$Ax_{0.6}$		
	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1

Casein[a]	500	500	500	500	500	500
Pollack liver oil[b]	40	40	40	40	40	40
Soybean lecithin[a]	40	40	40	40	40	40
PUFA[d]	10	10	10	10	10	10
L-arginine[c]	30	30	30	30	30	30
Cholesterol[c]	10	10	10	10	10	10
Vitamin mixture[e]	30	30	30	30	30	30
Mineral mixture[f]	60	60	60	60	60	60
α-Starch	50	50	50	50	50	50
Sucrose[c]	50	50	50	50	50	50
Glucose[c]	50	50	50	50	50	50
Glucosamine-HCl[c]	8	8	8	8	8	8
Sodium citrate[c]	3	3	3	3	3	3
Sodium succinate[c]	3	3	3	3	3	3
k-Carrageenan[g]	25	25	25	25	25	25
CMC	30	30	30	30	30	30
α-Cellulose	61	60.8	60	60.4	60.2	59.4
Ax[h]	0	0	0	0.6	0.6	0.6
Vitamin E[a]	0	0.2	1	0	0.2	1
Total	1000	1000	1000	1000	1000	1000

[a] Wako Pure Chemical Industries, Ltd. Osaka, Japan.

[b] Riken Vitamin, Tokyo, Japan.

[c] Nacalai Tesque, Kyoto, Japan.

[d] PUFA: (eicosapentaenoic acid) EPA 5 g and (docosahexenoic acid) DHA 5 g.

[e] Vitamin mix (30 g kg^{-1} diet) (Vitamin A and E free): 0.23 g *p*-aminobenzoic acid; 0.01 g biotin; 9.31 g inositol; 0.93 g nicotinic acid; 1.40 g Ca-pantothenate; 0.28 g pyridoxine-HCl; 0.19 g riboflavin; 0.09 g thiamine-HCl; 0.09 g menadione; 0.03 g cyanocobalamine; 0.002 g calciferol; 3.46 g stay-C; 0.02 g folic acid; 13.96 g choline choride.

[f] Mineral mix (60 g kg^{-1} diet): 13.98 g K_2HPO_4; 19.11 g $Ca_3(PO_4)_2$; 21.36 g $MaSO_4.7H_2O$; 5.55 g $NaH_2PO_4.2H_2O$.

[g] Sigma-Aldrich, St. Louis, MO, USA.

[h] Ax: Carophyll® Pink containing 10% astaxanthin made by DSM Nutrition, Japan.

Table 3-2 Chemical analysis of the experimental diets.

Compositions	Ax_0			$Ax_{0.6}$		
	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1
Moisture (%)	11.93	12.15	11.11	12.36	12.24	11.33
Crude lipid (%)	8.47	8.89	8.41	8.58	8.98	8.28
Crude protein (%)	46.16	45.89	46.3	46.24	45.83	45.95
Ash (%)	6.86	6.9	6.73	7.04	7.09	7.07
Ax content (g kg^{-1})	ND[a]	ND	ND	0.54	0.55	0.54
α-Toc content (g kg^{-1})	0.026	0.185	0.975	0.032	0.182	0.934

[a] ND, not detected

Table 3-3 Growth parameters and nutrient utilization in kuruma shrimp fed with an experimental diet for 56 days.[a]

Parameters[b]	Ax_0			$Ax_{0.6}$			Probability (P value)[c]		
	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	$P_{\alpha\text{-Toc}}$	P_{Ax}	$P_{Ax} \times P_{\alpha\text{-Toc}}$
FBW (g)	4.95 ± 0.11^{a}	5.27 ± 0.06^{abc}	5.38 ± 0.05^{bc}	5.23 ± 0.06^{abc}	5.55 ± 0.12^{c}	5.07 ± 0.07^{ab}	0.01	0.26	0.01
BWG (%)	31.10 ± 1.15^{a}	40.42 ± 1.84^{ab}	40.57 ± 3.37^{ab}	37.42 ± 2.12^{ab}	43.61 ± 3.25^{b}	30.11 ± 1.94^{a}	0.02	0.88	0.01
SGR (%)	0.45 ± 0.03^{a}	0.56 ± 0.02^{ab}	0.56 ± 0.04^{ab}	0.53 ± 0.03^{ab}	0.60 ± 0.04^{b}	0.44 ± 0.03^{a}	0.02	0.96	0.01
FI (g)	0.17 ± 0.72	0.19 ± 0.01	0.2 ± 0.01	0.16 ± 0.01	0.2 ± 0.02	0.16 ± 0.01	0.01	0.75	0.08
FCR	7.66 ± 0.97	7.49 ± 1.12	6.49 ± 0.90	7.83 ± 0.97	7.09 ± 1.01	6.33 ± 0.31	0.04	0.64	0.74
Survival (%)	93.33 ± 3.33	100 ± 0.00	96.67 ± 3.33	100 ± 0.00	96.67 ± 3.33	96.67 ± 3.33	0.22	0.63	0.78
IP (d)	0.33 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	0.33 ± 0.02	0.31 ± 0.03	0.25 ± 0.04	0.66	0.06	0.01

[a] Data are expressed as mean ± S.E.M. from three groups. Data with different superscript letters in one row represent a significant difference from the control group ($P < 0.05$).

[b] FBW, final body weight; BWG, body weight gain = [(final weight - initial weight)/initial weight × 100]; SGR, specific growth rate = [(Ln final weight - Ln initial weight)/duration × 100]; FI, feed intake = total feed intake (g)/number of shrimp in each tank/duration; FCR, feed conversion ratio = dry weight of feed consumed (g)/live weight gain of shrimp (g); IP, intermolt period. IP(d) = $\sum T/\sum N$.

[c] Significant effects determined by two-way ANOVA.

Table 3-4 Whole-body proximate analysis (% dry matter basis, except moisture) of kuruma shrimp fed with an experimental diet for 56 days.[a]

Parameters	Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	$P_{α\text{-Toc}}$	P_{Ax}	$P_{Ax} \times P_{α\text{-Toc}}$
Moisture (%)	77.31±1.03	77.55±2.01	78.43±1.81	78.49±0.04	77.38±1.99	78.28±2.65	0.89	0.85	0.92
Crude protein (%)	67.69±1.05	68.65±0.19	66.29±1.07	66.48±1.45	68.26±2.29	67.59±1.20	0.49	0.93	0.65
Crude lipid (%)	2.46±0.59	2.82±0.94	2.74±0.33	2.65±0.75	2.94±0.42	2.64±0.60	0.01	0.17	0.09
Ash (%)	14.07±0.65	16.26±0.38	18.64±0.44	18.14±0.52	17.14±0.30	19.06±0.18	0.07	0.13	0.24

[a] Data are expressed as mean ± S.E.M. from three groups.

[b] Significant effects determined by two-way ANOVA.

Table 3-5 Color parameters for cooked kuruma shrimp fed with an experimental diet for 56 days.[a]

Parameters[c]		Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
		α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	α-Toc_0	α-$Toc_{0.2}$	α-Toc_1	$P_{α\text{-Toc}}$	P_{Ax}	$P_{Ax} \times P_{α\text{-Toc}}$
Head	L^*	55.36±0.41^{ab}	57.89±0.77^{bc}	60.61±0.55^{c}	55.23±0.89^{ab}	52.94±0.57^{a}	53.23±0.71^{a}	0.06	0.01	0.01
	a^*	12.88±0.69	12.54±1.00	13.61±0.51	18.52±0.83	19.28±1.12	19.00±0.64	0.76	0.01	0.69
	b^*	19.58±0.24	21.78±1.01	21.31±0.43	22.90±0.53	23.88±1.04	23.48±0.97	0.15	0.01	0.68
Carapace	L^*	59.37±0.55	61.09±0.59	61.33±1.11	56.02±0.36	55.38±0.97	56.05±0.54	0.43	0.01	0.27
	a^*	12.19±0.11	13.53±0.37	14.04±0.61	16.76±0.27	17.57±0.89	16.75±0.27	0.10	0.01	0.20
	b^*	20.33±0.46^{a}	23.59±0.71^{bc}	22.29±0.11^{ab}	22.04±0.19^{ab}	23.47±0.42^{bc}	24.97±0.86^{c}	0.07	0.01	0.01
Muscle	L^*	66.15±0.62	66.45±0.79	68.51±0.66	64.02±0.70	62.06±0.97	63.14±0.47	0.14	0.01	0.11
	a^*	8.08±0.43^{a}	7.89±024^{a}	9.16±0.81^{a}	11.36±0.49^{b}	13.31±0.28^{b}	11.89±0.20^{b}	0.14	0.01	0.03
	b^*	16.81±0.35	18.58±0.61	19.58±0.65	17.69±0.35	18.63±1.11	17.13±0.83	0.13	0.36	0.06

[a] Data are expressed as mean ± S.E.M. from three groups. Data with different superscript letters in one row represent a significant difference from the control group ($P < 0.05$).

[b] Significant effects determined by two-way ANOVA

[c] L^*, lightness; a^*, redness; b^*, yellowness

Table 3-6 Total astaxanthin content of kuruma shrimp fed with an experimental diet for 56 days.[a]

Parameters	Ax_0	$Ax_{0.6}$	Probability (P value)[b]

		α-Toc$_0$	α-Toc$_{0.2}$	α-Toc$_1$	α-Toc$_0$	α-Toc$_{0.2}$	α-Toc$_1$	$P_{\alpha\text{-Toc}}$	P_{Ax}	$P_{Ax} \times P_{\alpha\text{-Toc}}$
	WB[c]	13.18±0.02[a]	19.31±0.05[b]	13.75±0.05[a]	27.31±0.05[c]	46.98±0.14[d]	63.90±0.44[e]	0.01	0.01	0.01
Total	Carapace	45.06±0.02[a]	60.29±0.18[b]	47.44±0.28[a]	330.69±2.23[c]	393.71±1.29[d]	389.29±2.14[d]	0.01	0.01	0.01
Ax	Muscle	27.39±0.03[a]	29.35±0.40[b]	31.34±0.09[c]	135.52±0.17[d]	154.86±0.02[e]	203.57±0.27[f]	0.01	0.01	0.01
	Head	31.83±0.05[a]	45.91±0.26[c]	37.69±0.13[b]	224.96±0.89[d]	272.32±0.74[e]	282.13±0.35[f]	0.01	0.01	0.01
Yield (%) of different body components										
	Carapace	12.03±1.94	10.43±0.16	10.29±0.22	10.19±0.24	10.89±0.67	10.94±0.22	0.32	0.32	0.29
	Muscle	42.24±1.59	44.98±0.88	44.69±0.79	45.55±0.70	44.78±0.18	44.64±0.45	0.52	0.18	0.12
	Head	45.74±0.52	43.81±0.25	45.02±0.89	44.27±0.59	44.34±0.76	44.42±0.35	0.82	0.73	0.31

[a] Data are expressed as mean ± S.E.M. from three groups. Data with different superscript letters in one row represent a significant difference from the control group ($P < 0.05$).

[b] Significant effects determined by two-way ANOVA.

[c] WB, whole body

Table 3-7 Digestive enzyme activity of kuruma shrimp fed with an experimental diet for 56 days.[a]

Parameters	Ax$_0$			Ax$_{0.6}$			Probability (P value)[b]		
	α-Toc$_0$	α-Toc$_{0.2}$	α-Toc$_1$	α-Toc$_0$	α-Toc$_{0.2}$	α-Toc$_1$	$P_{\alpha\text{-Toc}}$	P_{Ax}	$P_{Ax} \times P_{\alpha\text{-Toc}}$
Protease	0.74±0.02	0.97±0.05	0.95±0.15	0.89±0.10	1.49±0.03	1.04±0.09	0.01	0.01	0.09
Lipase	0.03±0.01[a]	0.04±0.01[a]	0.05±0.01[a]	0.08±0.01[a]	0.18±0.01[b]	0.19±0.03[b]	0.01	0.01	0.02
Amylase	0.20±0.04	0.21±0.02	0.22±0.01	0.33±0.04	0.35±0.01	0.37±0.01	0.53	0.01	0.88

[a] Data are expressed as mean ± S.E.M. from three groups. Data with different superscript letters in one row represent a significant difference from the control group ($P < 0.05$).

[b] Significant effects determined by two-way ANOVA.

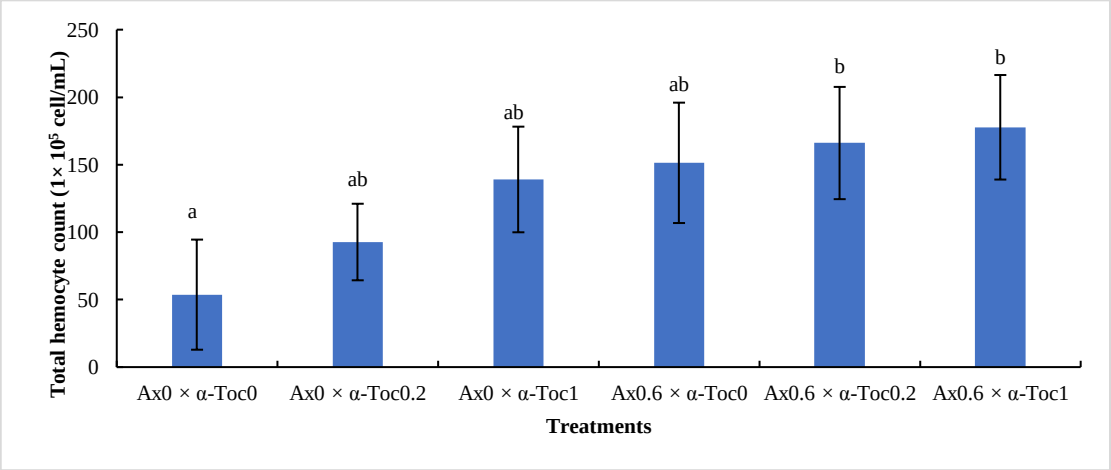

Fig. 3-1. Total hemocyte count of kuruma shrimp fed with an experimental diet for 56 days after being exposed to freshwater. Data are expressed as mean ± S.E.M. from three groups. Data with different letters represent a significant difference from the control group ($P < 0.05$).

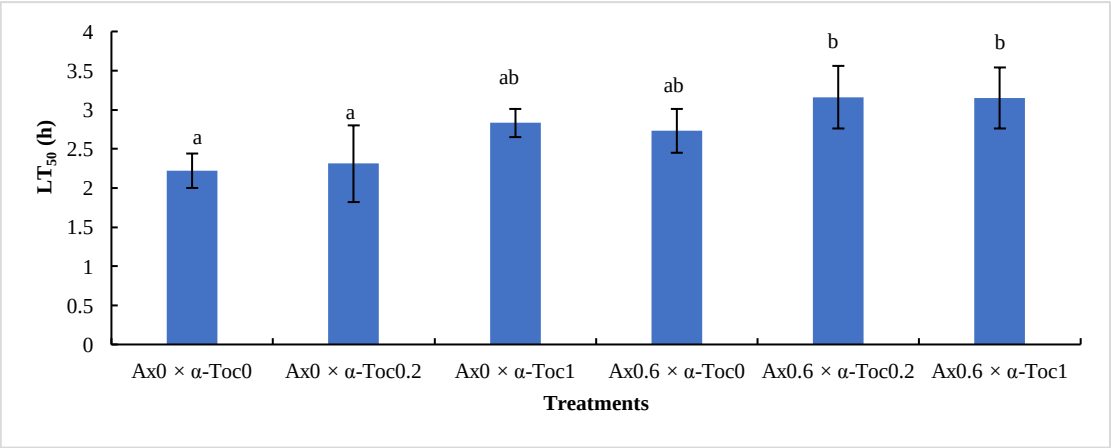

Fig. 3-2. LT_{50} (h) of kuruma shrimp fed with an experimental diet for 56 days, after being exposed to freshwater. Data are expressed as mean ± S.E.M. from three groups. Data with different letters represent a significant difference from the control group ($P < 0.05$).

Study 4: **Interactive Effects of Dietary Astaxanthin and Cholesterol on the Performances of Kuruma Shrimp, *Marsupenaeus japonicus***

Abstract

A 56-day feeding trial was carried out to evaluate the interactive effects of chemically synthesized astaxanthin (Ax) and cholesterol (CHO) on the performances of kuruma shrimp juveniles. Six experimental diets containing 2 levels of Ax (0 and 0.6 g kg^{-1} diet) and 3 levels of CHO (0, 6, and 20 g kg^{-1} diet) were formulated in 2 × 3 factorial design. The results indicated that the interaction between dietary CHO and Ax were not significant on survival, growth performances, feed utilization, body proximate analysis, immune response, and fresh water stress test. Only dietary Ax displayed significant difference in growth performances (final body weight, body weight gain, and special growth rate), and the highest values were recorded by the group fed with $Ax_{0.6}$ × CHO_6 combined diet. Furthermore, dietary CHO had significant differences in survival, feed utilization (feed intake and feed conversion ratio). Significantly better pigmentation performances and Ax contents of different parts of juveniles (whole body, muscle, carapace, and head) were observed in higher Ax ($Ax_{0.6}$) supplemented groups. Meanwhile, interactions of dietary Ax and CHO were also found on color reading parameters and whole-body Ax contents. In addition, juveniles fed with the diet containing Ax at 0.6 g kg^{-1} diet in addition to CHO at 0, 6 and 20 g kg^{-1} diet showed better performances on digestive enzyme activities (protease, lipase, and amylase) of hepatopancreas than 0 g Ax kg^{-1} diet supplemented with CHO at 0, 6 and 20 g kg^{-1} diet. Although the interactive effect was only found in the content of saturated fatty acid in neutral lipid, dietary Ax and CHO significantly altered the composition of fatty acid in kuruma shrimp body. It was concluded that dietary Ax and CHO functioned interactively. The addition of CHO can significantly promote the Ax deposition in the tissues which may enhance the positive effect of dietary Ax.

Keywords: Astaxanthin; Cholesterol; Interaction; Immune response; Growth; Coloration; Kuruma shrimp

Introduction

Kuruma shrimp culture has been practiced in Japan for many decades. Consequently, aquaculture is the only viable way of bridging the gap between demand and supply of this high-value seafood (Bulbul et al., 2014). High-density shrimp farming systems are widely used by farmers now. However, kuruma shrimps reared under intensive culture conditions with artificial diets are subject to loss of their natural colors (Chien and Jeng, 1992). Maintenance of the natural skin pigmentation is considered very important from a commercial point of view which directly correlates with product market price and selection by consumers (Shahidi and Brown, 1988). The coloration change of crustaceans can be due to physiological or morphological mechanism (Wade et al., 2012). The influence of carotenoid availability in the diet or from culture environment, photoperiod, light intensity, and temperature are considered some of the physiological mechanisms (Rao, 1985). Color changes induced by physiological mechanisms are often rapid, reversible, and rhythmic. In contrast, the morphological mechanisms are considered to be slower and more permanent which involve quantitative modifications of exoskeletal and hypodermal layer pigment concentration or composition (Wade et al., 2012). Niu et al. (2012) illustrated that dietary supplementation of carotenoids can improve or correct the color of penaeids for a better market price. The desired coloration can be achieved by including astaxanthin (Ax), canthaxanthin, zeaxanthin, β-carotene, and lutein (Chien and Shiau, 2005; Ju et al., 2011; Niu et al., 2014). Okada et al. (1994) indicated that Ax is the predominant pigment (86-98% of total carotenoids) in penaeids and other crustaceans. Moreover, main pigment usually being Ax in the free or esterified form is found in crustaceans (Niu et al., 2012). Although Ax is widely present in crustaceans, shrimps are unable to biosynthesize it (Meyers and Latscha, 1997). On the other hand, crustaceans are able to convert different carotenoids into Ax (Schiedt et al., 1993). According to previous studies, dietary Ax was more efficient than other carotenoids in improving pigmentation of shrimp (Chien and Jeng. 1992). Besides pigmentation properties of Ax, increasing attention is being directed towards defining its physiological functions in aquatic animals. Such functions include being the precursor of vitamin A which plays a prominent role in shrimp development and differentiation as well as an antioxidant which is closely associated with stress resistance performances (Christiansen et al., 1995; Linan-Cabello et al., 2002).

Since the market price of commercial synthetic Ax is high, this can significantly influence feed and production costs. Previous studies have investigated optimal levels of synthetic Ax for pigmentation of kuruma shrimp. Yamada et al. (1990) observed that pigmentation was increased in *Penaeus japonicus* fed diets with increasing synthetic Ax levels up to 200 mg kg^{-1} diet in an 8-wk trial. While Wang et al. (2018) showed that optimum diet supplementation level of the Ax to enhance the performance of juvenile kuruma shrimp is around 400 mg kg^{-1} diet. Furthermore, certain dietary additives such as vitamin E, cholesterol, fiber, lutein, zeaxanthin, and phytosterol are known to influence absorption of carotenoids in animals (Yeum and Russel, 2002). To improve the Ax efficiency is the more cost-effective means of achieving the desire coloration (Niu et al., 2014).

Cholesterol (CHO) is an important sterol which serve as a precursor for many physiologically active compounds such as sex and molting hormones, adrenal corticoids, bile acids, and vitamin D (Sheen, 2000). Crustaceans are incapable of de novo production of sterols (Sheen et al., 1994; Teshima and Kanazawa, 1971). Dietary CHO is essential for good growth and high survival in crustaceans (Sheen, 2000). Additionally, CHO has been found to have the highest nutritive value among sterols for juvenile kuruma shrimp (Teshima, 1997). Since carotenoids are lipid-soluble compounds, the amount and type of fat present in the diet may influence carotenoid bioavailability (Regost et al., 2004). An improvement in Ax deposition and coloration of Atlantic salmon was found by increasing dietary CHO (Buttle, 2000). Niu et al. (2012) also indicated that the supplement of CHO could positively enhance the efficiency of Ax in the commercial diet of *Penaeus monodon*.

Most of the available reports describing the metabolism of Ax in aquatic animals were assessed on salmonids (Lim et al., 2017). The mechanism by which high dietary CHO increases the bio-accessibility of Ax in shrimp is still unknown. Therefore, the current study was designed to assess whether dietary CHO can enhance or interfere with Ax absorption in juvenile kuruma shrimp, and to investigate the interactions between dietary Ax and CHO on the growth performance, digestibility, pigmentation, immune response, stress response, and fatty acid analysis in juvenile kuruma shrimp.

Materials and Methods

Test Shrimp and Experimental System

Juveniles of kuruma shrimp (*Marsupenaeus japonicus)* were obtained from Matsumoto Suisan, Miyazaki, Japan, and transported to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University. The juveniles were maintained in three 500-L black polyethylene circular tanks without sand bottom at the ambient water temperature (26 ± 0.5°C) fed with commercial diet (Higashimaru Feeds Ltd., Kagoshima, Japan) for one week. After acclimatization to laboratory conditions, 10 kuruma shrimp juveniles (4.38 ± 0.03 g initial wet weight) were randomly selected from stock tanks and were transferred to experimental tanks for feeding trial. The feeding trial was conducted in eighteen 32-L black rectangular polyvinyl chloride (PVC) tanks filled with 26-L seawater for 56 days. Sand (approximately 15 mm thickness) were placed on the bottom of each tank. Each tank was covered with a net and black plastic film to prevent shrimp from jumping out and to minimize disturbances. Throughout the feeding trial, the tanks were continuously aerated, while the salinity of the rearing water was maintained at 34 ± 0.5 ppt, with a flow rate of 60 mL min^{-1}.

Diet Preparation

Six experimental diets were formulated with two levels of Ax at 0 and 0.6 g kg^{-1} diet (Ax_0 and $Ax_{0.6}$) and three levels of CHO at 0, 6, and 20 g kg^{-1} diet (CHO_0, CHO_6 and CHO_{20}) in a factorial design (2 × 3) as shown in Table 4-1. Casein was used as the major protein source. Pollack liver oil and CHO were used as the major lipid sources, while, soybean lecithin was used as the phospholipids source. The mineral and vitamin mixtures were added according to Moe et al. (2004) and Alam et al. (2004) with slight modifications. Gluten served to bind the diets. Other ingredients were added to meet the nutritional and growth requirements for juvenile kuruma shrimp.

Dry ingredients of the experimental diets were mechanically mixed to insure homogeneity. Lipid sources and fat-soluble components were premixed and then added to the dry ingredients until mixed well. Distilled water was added at a quantity of 25-30% of the dry ingredients, and

the pH was adjusted to 7.0-7.5 using 1N sodium hydroxide. After thoroughly blended, the mixture was then molded into an appropriate diameter (1.2-2.2 mm) to the prepare pellets using a single-screwed mincer (ROYAL Inc., Tokyo, Japan). The pellets were then dried in a dryer mechanical convection oven (DK400, Yamato Scientific, Tokyo, Japan) at 60°C until the moisture content was reduced to 10%. The dried pellets were steamed at 100°C for 1 min in a cylindrical steamer to improve their water stability, and were stored at -20°C until use.

Juveniles Feeding Trial

Juveniles were fed twice daily with test diets at a ration amount of 8-10% of body weight. Daily proportion was divided into 20% at 08:00 am and 80% at 17:00 pm. Every morning, uneaten diets were recorded using pellet counts that converted to gram weight based on average pellet weight using well-established methods (Wade et al., 2017) to calculate feed intake (FI) and feed conversion ratio (FCR). Then siphoning the uneaten diets together with fecal matters from tanks. Every 14 days, juveniles were bulk-weighted, and the survivors in each tank were counted. At the same time, the tank and sand were cleaned and filled with fresh rearing water.

Sample Collection and Biochemical Analysis

At the end of the feeding trial, all treatment groups were fasted for 24 h prior to final sampling. The total numbers of survivors and individual body weight from each tank were measured using following formulae:

Body weight gain (BWG, %) = [(final weight-initial weight)/initial weight] × 100

Specific growth rate (SGR, % day^{-1}) = [(Ln final weight-Ln initial weight)/duration] × 100

Feeding intake (FI, g/shrimp/56 days) = total feed intake/shrimp number/duration

Feed conversion ratio (FCR) = dry weight of feed consumed (g)/live weight gain (g)

Survival (%) = (final number of shrimp/initial number of shrimp) × 100

Hepatopancreas index (HSI, %) = hepatopancreas weight/body weight × 100

Three juveniles from each tank were obtained for immunological analysis. A quantity of 200 µL haemolymph was collected from the ventral sinus of shrimp with 800 µL of sterile

anticoagulant (NaCl 29.22 g L^{-1}, EGTA 3.8 g L^{-1}, Hepes 2.38 g L^{-1}, and *L*-cysteine 5 g L^{-1} of stock solution) using a 1 mL syringe (Terumo Corp., Tokyo, Japan). The mixture was immediately placed into a vial, chilled on ice and transported to the laboratory for further analysis.

Proximate analysis of the diets and whole body of shrimp were analyzed by standard AOAC methods (AOAC, 1990), including moisture by oven-drying at 110°C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550°C for 4 h.

Astaxanthin Analysis

The shrimp samples were freeze dried and then crushed to form a powder. The samples were then placed into a 50 mL polypropylene centrifuge tube for Ax analysis following the method of Masako et al. (2010), with slight modifications. The Ax was then extracted with chloroform by ultrasonic mixing for 10 min and was centrifuged at 4°C and at 11800 rpm for 15 min. The extracted Ax was transferred to flasks and dried in rotary vacuum evaporator (Eyela SB 1100, CCA 1111). The sample extract was then dissolved in 10 mL acetone solution. A volume of 0.05 M Tris-HCl buffer (pH 7.0) 6 mL and cholesterol esterase solution (60 unit mL^{-1}) 600 µL was added to the extract, and the enzyme reaction allowed to complete for 120 min at 37°C. After the enzyme reaction, the solution was transferred to a 250 mL separatory funnel, partitioned with 10 mL petroleum ether. The solution was washed three times with 0.01 g $NaSO_4$ to remove residual acetone. Then, using the rotary vacuum evaporator again, the solvent was mobilized and filtered through a 0.2 µm Millipore filter and stored in brown vials. Ax was analyzed by high-performance liquid chromatography (HPLC), using a shim-pack VP-ODS column (150 mmL. × 4.6 mmL.D.) and a SPD-M20A detector at 475 nm. The operational conditions were: mobile phase, 0.05% trifluoracetic Acid/methanol = 3/97 (v/v) and solvent flow rate, 1.2 mL min^{-1}; injection volume, 10 µL. This system was controlled by a chromatographic data system (Scientific Information Services Corporation), which also integrated the areas under the peaks. The standard was prepared using 8% Ax contained in

Carophyll Pink®. The standard was prepared for five levels (0.05-20 mg L^{-1}) using 8% Ax contained in Carophyll Pink®. And the standard curve was presented by the equation Y = 60595x - 9081 (R^2 = 0.99).

Fatty Acid and Cholesterol Analysis

Fatty acid analysis was analyzed using a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with a flame ionization detector using an Omegawax 320 column (Supelco, Inc., Japan). Fatty acid methyl esters were prepared using the shrimp body according to the method of Bligh and Dyer (1959) and then further separated into neutral lipid (NL) and polar lipid (PL) fractions by Sep-pak silica cartridge (Waters Corporation, Milford, Massachuseetes, USA) as described by Juaneda and Rocquelin. (1985). Both fractions were analyzed to quantify the content of CHO from lipid classes analyzed by MK-6s Iatroscan equipped with a flame ionization (Iatron Lab., Japan) (Thongrod and Boonyaratpalin, 1988). Peaks were integrated with a chromatopac C-R8A (Shimadzu, Japan). Methylating rest of the NL and PL by adding calculated amount of C23:0, respectively. Keeping samples into the heat reactor for 1 h with 1 mL boron trifluoride and 0.5 mL dichloromethane. After that, mixing well with 0.5 mL NaCl and 1 mL hexane, and taking the upper layer for further fatty acid analysis.

Analysis of Digestive Enzymes

The hepatopancreas samples were collected in 1 h after the final sampling to guarantee maximum activity of digestive enzymes. The hepatopancreas of shrimp were dissected out, weighted and homogenized with shrimp saline shrimp saline solution (NaCl 28.4 g L^{-1}, $MgCl{\cdot}6H_2O$ 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 2 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 2.25 g L^{-1}, KCl 0.7 g L^{-1}, Glucose 1 g L^{-1}, Hepes 2.38 g L^{-1}) in ice cooled condition. The homogenate was centrifuged at 6000 rpm for 20 min at 4°C. After centrifugation, collecting the supernatant fluid. The samples were stored at -80°C until analysis.

Protease activity was analyzed with the method of Sigma's Non-specific Protease Activity Assay (Cupp-Enyard, 2008) with slight modifications. The enzyme assays were conducted

with using casein as the substrate and reacting with Folin-phenol reagent. The 0.5 mL of diluted enzymatic extract for each sample were incubated at 37°C for 10 min with adding 5 mL of 0.65% casein. Then stopping the reaction by adding 5 mL 110 mM trichloroacetic acid (TCA) for 30 min. Filter the mixture using a 0.45 μm syringe filter, and then 5 mL 500 mM sodium carbonate solution and 1 mL of 0.5 mM Folin's reagent were added and incubated at 37°C for another 30 min. After incubating, using the 0.45 μm syringe filter to filter again for the better results measured by the spectrophotometer (Hitachi High-Technologies Corportaion, Japan) at A_{660} versus blank.

Amylase activity was analyzed with the method of Shiu et al. (2015) with slight modifications. Incubate the tubes added with 0.5 mL enzyme solution for 3-4 min to achieve temperature equilibration. At timed intervals, add 0.5 ml starch solution and incubate exactly 3 min at 25°C. At timed intervals add 1 mL dinitrosalicylic acid color reagent to each tube and incubate another 5 min in a boiling water bath. Then cool to room temperature and add 10 mL distilled water. Mix well and read by spectrophotometer at A_{540} versus blank.

Lipase activity was analyzed with the method of Mustafa et al. (2016) with slight modifications. The enzyme assays were conducted by using olive oil emulsion as the substrate, and produced fatty acid was extracted by isooctane, then the extracted fatty acid was determined by copper soap colorimetry measurement. The olive oil emulsion was prepared by mixing 50 mL of olive oil and 150 mL 4% polyvinyl alcohol solution in a homogenizer. The reaction mixture containing 2 mL of olive oil emulsion, 2.5 mL of 20 mM phosphate citrate buffer (pH 7.5) and 0.5 mL enzyme solution was incubated at 40°C for 15 min. The reaction was then immediately stopped after incubation, by adding 1 mL 6 M HCl and 6 mL 95% ethanol. After mixing well, collecting 1 mL of the supernatant fluid and adding 4 mL of isooctane and 1 mL 5% cupric acetate pyridine reagent (pH 6.1) for reading by the spectrophotometer at A_{714} versus blank.

Color Measurement

The plastic bags of samples (six shrimp/bag) were placed in a boiling water bath for 3 min. The bags were then cooled in tap water (about 5°C) in the dark following the method of Ju et

al. (2011). And Ando et al. (2014) stated that kuruma shrimp cooked at 100°C and stored at 5°C showed better color development rate. Color parameters (L^* = lightness, a^* = redness, b^* = yellowness) were obtained with a colorimeter (Chroma Meter CR400, Konica Minolta Sensing Inc., Osaka, Japan) according to Nickell and Bromage (1998). Three parts including head, body and muscle (shell removed) of shrimp were read by the colorimeter.

Immune Response

The total haemocyte count (THC) method described by Itami et al. (1998) was used in current study. Phenoloxidase activity (PO) of haemolymph was measured spectrophotometrically by recording the formation of dopachrome produced from *L*-DOPA with the method described by Hernandez-Lopez et al. (1996). Lysozyme activity (LY) of hepatopancreas was determined with turbidimetric assay (Lygren et al., 1999), using *Micrococcus lysodeikticus* (lyophilized cell, Sigma, USA) as specific substrate. A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min. Lipid oxidation levels of hepatopancreas were determined based on the malondialdehyde (MDA) levels generated by oxidation of polyunsaturated fatty acids. In the presence of thiobarbituric acid, malondialdehyde started producing colored TBARS measured at 532 nm (Buege and Aust, 1978).

Fresh Water Stress Resistance Test

After feeding trial, five similar size of shrimp from each tank were chosen and challenged with fresh water to investigate the tolerance against stressors and evaluate the efficiency of test diets. 10 L experimental glass bottles filled with 8 L tap water were dechlorinated by aeration for 24 h before starting the stress test. During the stress resistance test trial, mortality was monitored every 20 min. Cumulative mortality (%) was transformed into common $\log_{10}$ (survival) to fit a regression line and the mean was calculated by the regression equation (Dawood et al., 2015a; Yokoyama et al., 2005). The lethal time of 50% mortality was expressed as the LT_{50}.

Statistical Analysis

All data were presented as mean values ± standard error of mean (S.E.M., n=3). The statistical analysis was performed by using an analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA) after exploring the normality and homogeneity of data. Data from each group were compared using Turkey Kramer test. Differences between treatments were considered significant when $P < 0.05$. Two-way ANOVA was employed to test the effects of dietary CHO levels and Ax levels, as well as their interactions. If no interaction occurred, One-way ANOVA was attributed to each individual factor to evaluate the inclusion level of each factor (Dawood et al., 2015b; Michael et al., 2006).

Results

Growth Performances and Nutrient Utilization

Growth performance, survival, and feed utilization of the test shrimp fed with experimental diets for 56 days were given in Table 4-3. The interaction between dietary CHO and Ax were not significantly different on all the parameters. Only dietary Ax showed significance in final body weight (FBW), body weight gain (BWG), and specific growth rate (SGR). Dietary CHO was significantly differently in feed intake (FI) and survival rate. Both dietary CHO and Ax showed significant differences in feed conversion ratio (FCR). Neither dietary CHO nor Ax were the significantly different in terms of hepatopancreas index (HSI). By dietary CHO and Ax supplementations, the fastest growth performance (FBW, BWG, and SGR) was found in $Ax_{0.6} \times CHO_6$ group.

Whole Body Proximate Analysis

The whole body proximate analysis of kuruma shrimp fed with experimental diets for 56 days was shown in Table 4-4. There were no interactive effects between dietary Ax and CHO on shrimp whole body proximate analysis. Crude protein and ash were found to be similar to those of the control group in all treatments. The crude lipid increased with increasing CHO supplementation.

Pigmentation of Shrimp

The color parameters of cooked whole shrimp body read by colorimeter were shown in Table 4-5. Dietary CHO, Ax supplementations and interaction between the two additives were not significantly different in lightness (L^*) of the head. However, dietary CHO, Ax supplementations and interaction between these two additives were significantly different in terms of redness (a^*) of head, yellowness (b^*) of head, redness (a^*) of body, yellowness (b^*) of body, and redness (a^*) of muscle. Only dietary Ax was statistically significant in lightness (L^*) of body. Only the interaction between the two additives was significantly different in lightness (L^*) of muscle. Both dietary CHO and Ax showed significant differences in terms of yellowness (b^*) of muscle.

Only $Ax_{0.6} \times CHO_0$ group was significantly different ($P < 0.05$) from $Ax_{0.6} \times CHO_{20}$ group on the lightness (L^*) of muscle. The redness (a^*) of head, shell, and muscle in all higher Ax ($Ax_{0.6}$) supplemented groups showed significantly higher ($P < 0.05$) values than the ones in all the lower Ax (Ax_0) supplemented groups. And the highest redness (a^*) values were given by $Ax_{0.6} \times CHO_6$ group, and were significantly different from the other groups on all the parts of shrimp. The yellowness (b^*) of head and body showed an increasing trend with the increased CHO supplementation level in lower Ax (Ax_0) three groups. Moreover, the $Ax_{0.6} \times CHO_6$ group showed the highest yellowness (b^*) values on all the parts of shrimp.

Determination of Astaxanthin

Total Ax content (free Ax + esterified Ax) in whole body, shell, muscle, and head of kuruma shrimp fed with experimental diets for 56 days was shown in Table 4-6. Dietary CHO,

Ax supplementations and interaction between these two additives were significant factors ($P < 0.05$) on the total Ax content in different parts of shrimp. Comparing to the lower Ax (Ax_0) supplementation three groups, total Ax content of different parts of shrimp body in higher ($Ax_{0.6}$) Ax supplementation three groups showed significantly higher values ($P < 0.05$). In the higher Ax ($Ax_{0.6}$) supplementation three groups, the significantly higher values were given by the $Ax_{0.6} \times CHO_6$ group in different parts of shrimp.

Digestive Enzyme Activities

Digestive enzyme activities of kuruma shrimp fed with experimental diets for 56 days was shown in Table 4-7. Dietary CHO, Ax supplementations and interaction between two additives were significant factors ($P < 0.05$) on lipase activity. Only Ax was a significant factor on protease and amylase activities.

Comparing to the lower Ax (Ax_0) supplementation three groups, protease activity for higher ($Ax_{0.6}$) Ax supplementation three groups showed higher values. The similar better performance was given by higher ($Ax_{0.6}$) Ax supplementation three groups on amylase activity when compared to the lower Ax (Ax_0) supplementation three groups. Either CHO or Ax supplemented groups showed significantly higher ($P < 0.05$) values than the control group on lipase activity. And there were no significantly differences ($P > 0.05$) among these two additives supplemented groups.

Fatty Acids Analysis

Fatty acids analysis of the test shrimp whole body fed with experimental diets for 56 days were given in Table 4-8 and Table 4-9. For neutral lipid, dietary CHO and the interaction between dietary CHO and Ax were significantly different in the contents of ∑SFA. Both dietary CHO and Ax showed significant differences in ∑MUFA and ∑PUFA. Only dietary Ax showed significant differences in DHA (22:6n-3). For polar lipid, dietary Ax and the interaction between dietary CHO and Ax had significant differences in terms of EPA (20:5n-3). Dietary CHO and Ax were both significantly different on ∑SFA, ∑MUFA, and ∑PUFA.

Non-specific Immune Response

Immune response of kuruma shrimp are presented in Table 4-10. The total haemocyte count (THC), phenoloxidase activity (PO), malondialdehyde levels (MDA), and lysozyme activity (LY) were not significantly affected by dietary CHO supplementation. And there were no interactions detected between various treatments by dietary CHO and Ax. Only dietary Ax is the significant factor on THC, LY, and MDA parameters ($P < 0.05$).

Fresh Water Stress Test

The LT_{50} values for the freshwater stress tolerance test of kuruma shrimp fed with experimental diets for 56 days were illustrated in Fig. 4-1. Although no interactive effect was found on LT_{50} of kuruma shrimp exposed to the freshwater, dietary CHO and Ax were significant factors on this parameter. Comparing to the lower Ax (Ax_0) supplementation three groups, higher ($Ax_{0.6}$) Ax supplementation three groups showed better performances.

Discussion

The studies on the dietary factors that might enhance or interfere with Ax absorption in fish have been reported previously by Chimsung et al. (2014). But interactions between conditional indispensable dietary factors and Ax, especially on shrimp, are still unrevealed. Under these circumstances, it is important to investigate the interactions between functionally related dietary factors that may improve the Ax efficiency or to identify alternative, more cost-effective way of achieving the desire coloration.

Both of the Ax and CHO play important roles on growth performance, feed utilization and survival of crustaceans (Kanazawa et al. 1971; Kumar et al., 2009; Niu et al., 2012), where both of them are also considered as conditional indispensable ingredients for kuruma shrimp

(Chien et al., 2005; Teshima, 1997; Yamada et al., 1990). Based on the results of the current study, only dietary Ax supplementation is the significant factor on growth parameters (FBW, BWG, and SGR) of kuruma shrimp. These results are consistent with the findings reported by Chien and Shiau, (2005) who also found that dietary Ax supplementation can significantly affect kuruma shrimp growth performance. Nevertheless, growth parameters increased when dietary Ax levels increased, this might be partly explained by the increased trend of feed utilizations which is consistent with previous studies on Ax on *Penaeus monodon* (Niu et al., 2012). Teshima, (1997) suggested that supplementation of 1 and 5 g kg^{-1} CHO significantly improved the weight gain of kuruma shrimp. Similar increasing trend of growth parameters can be found when dietary CHO levels increased to 6 g kg^{-1} diet and then decreased when the level up to 20 g kg^{-1} diet in current study. Similarly, Sheen (1994) reported that there was no significant difference in weight gain of *Penaeus monodon* fed diets containing 2-8 g kg^{-1} CHO. Furthermore, in his study, the diet containing 10 g kg^{-1} CHO had an adverse of toxic effect on shrimp growth. Different optimum dietary levels may due to the different composition of the basal ingredients which can significantly affect the absorption of CHO. Together with the previous studies, proper supplementation of CHO can affect the growth performance of kuruma shrimp (Deshimaru and Kuroki. 1974; Shudo et al., 1971; Teshima, 1997). Gastrointestinal absorption and metabolism of Ax are known to be strongly influenced by the presence of certain dietary CHO level (Lim et al., 2017). Combing with the results obtained by Niu et al. (2014) who found that not only 1 g kg^{-1} Ax but also the combination of 1 g kg^{-1} Ax and 10 g kg^{-1} CHO can significantly affect *Penaeus monodon* growth. Furthermore, Niu et al. (2012) showed that the combination of 1 g kg^{-1} Ax and 1% CHO showed better growth performance than the 1 and 2 g kg^{-1} Ax supplementation groups of black tiger shrimp. The functional relationship status of the Ax and CHO indicates an interactive relationship between them. However, there was no interaction between Ax and CHO on the growth parameters (FBW, BWG, and SGR) of kuruma shrimp in the current study. HSI of kuruma shrimp is not altered by neither Ax nor CHO in the current study which indicate that the dietary levels of Ax or CHO did not affect morphological index of kuruma shrimp. The 8 weeks survival rates of shrimp didn't show significant interaction between Ax and CHO in current study. Only the CHO was

found to be the factor which can significantly increase the mortality that happened in high CHO ($Ax_0 \times CHO_{20}$ and $Ax_{0.6} \times CHO_{20}$) supplementation groups. Similarly, in several studies Ax supplementation did not significantly affect shrimp survival and proper supplementation of CHO can promote the survival (Chien and Jeng, 1992; Ju et al., 2011; Liao et al., 1993; Sheen et al., 1994; Teshima, 1997). Mercer (1982) indicated that for most essential nutrients, there is a characteristic nutrient-response curve which increases to a point and then tend to level off. It is also considered that physiological responses to nutrients are graded. Combining with the phenomena in current study, the high level of dietary CHO which caused the negative growth and survival response may be a nutrient-response characteristic rather than toxicity.

Only CHO is the significant factor on crude lipid carcass analysis. These results are consistent with the findings reported by Niu et al. (2014) which showed that not only the shrimp in CHO combined with Ax supplementation group but also that combined with β-carotene group showed significantly higher lipid content than the same supplementation level of Ax or β-carotene but without CHO groups.

Cooked shrimp color directly correlates with the presence of Ax within hypodermal tissues (Boonyaratpalin et al., 2001; Menasveta et al., 1993). Within the exoskeleton and hypodermal tissue, Ax is often bound within a multimeric protein complex called crustacyanin (CRCN) (Wade et al., 2012). During cooking, the interaction between the Ax and CRCN is disrupted, releasing the distinct red coloration of shrimp. Color has successfully been quantified in shrimp using colorimeter, especially the *a** (redness) color parameters can well explain the color differences from the visual inspection of the cooked whole shrimp body by dietary supplemented α-Toc and Ax. By visual inspection of the head, shell, and muscle of cooked shrimp in the current study, the higher Ax ($Ax_{0.6}$) groups clearly shows the more pronounced red color than the pink color of shrimp fed with lower Ax (Ax_0) diets. The phenomena consist with the *a** (redness) values which significantly higher in higher Ax ($Ax_{0.6}$) groups than the ones in lower Ax (Ax_0) groups in the current study. And the highest redness (a*) values given by $Ax_{0.6} \times CHO_6$ group can also be explained by the Ax content of $Ax_{0.6} \times CHO_6$ group which showed significantly differ from the other groups on all the parts of shrimp. Niu et al. (2012) showed that the apparent digestibility coefficient (ADC) of *Penaeus monodon* fed Ax is quite high (> 98%), and CHO supplementation in the diet could not improve the ADC of Ax any

more. Further, dietary Ax showed low degree of retention efficiency of carotenoids and CHO supplementation can positively enhance the efficiency of Ax in *Penaeus monodon*. These results are consistent with the Ax content of carapace, muscle, and head of kuruma shrimp in current study, which significantly improved by CHO supplementation. Combining with current and previous studies, it may be inferred that dietary Ax can be digested effectively in gastrointestinal tract, but can't be resultantly deposited in tissues as pigmentation. And dietary CHO supplementation can significantly promote the Ax deposition in kuruma shrimp tissues.

The digestive enzyme activity in crustacean plays a central role in nutritional physiology and may directly or indirectly regulate growth, moulting cycle and complex dietary formulation (Lovett and Felder, 1990; Moullac et al., 1996). The hepatopancreas of shrimp combines the functions of the pancreas, intestine and liver that is responsible for the digestion processes such as digestive enzymes, absorption of digested material and metabolism of lipids, carbohydrates and minerals. The retention of nutrition can be directly or indirectly decided by the activity of digestive enzymes. In current study, dietary Ax supplementation is indicated as the significant factor for growth performances (FBW, BWG, and SGR) and digestive enzymes activities (protease, lipase, and amylase), respectively. Kuruma shrimp is believed to require more dietary protein for growth than other crustacean species (Bulbul et al., 2016). The higher level of protein enzyme activity obtained with diets containing Ax improved the digestion ability, which might in turn explain the better growth performance observed with $Ax_{0.6}$ groups. Dietary CHO, Ax supplementations and interaction between two additives were significant factors ($P < 0.05$) on lipase activity in current study. This effect also suggests that the difference in whole body crude lipid content of shrimp fed with Ax and CHO supplemented diets might be due to improved nutrition catabolism and digestion ability. Moreover, Ax are known as the lipid-soluble compounds. Therefore, the amount and type of fat present in shrimp tissues may influence Ax bioavailability (Regost et al., 2004). The better coloration performance and higher Ax content showed by $Ax_{0.6}$ groups might be due to strong elevation of lipase activity.

In crustacean exoskeleton and salmon muscle, Ax is predominantly esterified with one or two units of fatty acids (monoesters and diesters) or conjugated with proteins in order to provide stability to the molecule (Bjerkeng et al., 2000; Storebakken et al., 2004). Increased levels of dietary carotenoid resulted in the accumulation of carotenoid esters in curstaceans

(Boonyaratpalin et al., 2001; Yamada et al., 1990). Wade et al. (2017) also showed that Ax monoesters are enriched with saturated fatty acids, whereas Ax diesters are enriched with monounsaturated and polyunsaturated fatty acids in *Penaeus monodon*. The carapace of the spiny lobster *Panulirus japonicus* was found to contain 42% and 12% Ax diesters and monoesters, respectively. Moreover, it is well-known that carotenoids are lipid-soluble compounds, which are divided into more polar and non-polar subclasses and Ax is the more polar compound. Combining with previous studies, dietary Ax may induce the increasing of MUFA or PUFA in kuruma shrimp body, particularly in polar-lipid analysis. This is consistent with the result in present study where $Ax_{0.6}$ three groups showed higher MUFA and PUFA than the Ax_0 three groups for the polar lipid analysis. Niu et al. (2014) illustrated that the polar compound Ax may more easily be transferred since they are mainly located in the lipoprotein surface of the lipid droplets. The similar change is not found in the neutral lipid analysis. The accumulation of nonpolar lipid in the whole body of *Penaeus merguiensis* was related to the CHO level in the diet (Thongrod and Boonyaratpalin, 1998). Thus, dietary CHO is the significant factor for the neutral lipid analysis in the current study.

Different from the fish, crustaceans are highly dependent on cellular and humoral components of non-specific immune system (Adel et al., 2017). Crustacean haemocytes are thought to be functionally analogous to vertebrate leukocytes and involved primarily with the recognition and removal of foreign materials (Lee and Shiau, 2004). Dietary Ax can significantly affect the THC values in current study. Similarly, Flores et al. (2007) reported enhanced THC values in white shrimp *Litopenaeus vannamei* (Boone) juvenlies fed diets supplemented with Ax. PO plays a key role in immune defense of invertebrates and is used as a reliable indicator of immune status in shrimp (He et al., 2017). Although dietary Ax, CHO, and the interaction between these two additives didn't significantly alter the PO values, the PO values did appear to be higher due to the supplementation of CHO and Ax. Indicating that dietary CHO and Ax may contribute the production of phenoloxidase during the proPO system activiation. Furthermore, lysozyme activity and MDA have been used frequently as important indicators of non-specific immunity for crustaceans. Although no significant differences among treatments were detected on values of lysozyme and MDA under the current conditions, dietary Ax was shown as a significant factor on these two parameters. Niu et al. (2014)

indicated that MDA as an indicator of lipid peroxidation in shrimp fed Ax-containing diets were significantly lower than that of shrimp fed the basal diet. This suggests that dietary Ax may serve to protect the polyunsaturated fatty acids of tissues for shrimp under oxygen depletion stress. And it can be partly explained that synthetic Ax exhibits free radical scavenging, singlet oxygen quenching, and antioxidant activities which could probably positively affect animal and human health (Dose et al., 2016).

Conclusion

In conclusion, interactive effects by dietary CHO and Ax existed on growth parameters, lipase activity, pigmentation, and total Ax content in different parts of shrimp body. The addition of CHO can enhance the positive effects of dietary Ax. These enhanced effects were possibly due to the increasing levels of Ax found in whole body, muscle, carapace, and head tissues.

Table 4-1. Composition of the experimental diets (g kg^{-1}, dry matter basis).

[a] Wako Pure Chemical Industries, Ltd. Osaka, Japan.

Ingredients	Ax_0			$Ax_{0.6}$		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}
Casein[a]	500	500	500	500	500	500
Pollack liver oil[b]	40	40	40	40	40	40
Soybean lecithin[a]	40	40	40	40	40	40
PUFA[d]	10	10	10	10	10	10
L-arginine[c]	30	30	30	30	30	30
Vitamin mixture[e]	12	12	12	12	12	12
Mineral mixture[f]	86	86	86	86	86	86
α-Starch	50	50	50	50	50	50
Sucrose[c]	50	50	50	50	50	50
Glucose[c]	50	50	50	50	50	50
Glucosamine-HCl[c]	8	8	8	8	8	8
Sodium citrate[c]	3	3	3	3	3	3
Sodium succinate[c]	3	3	3	3	3	3
Gluten	55	55	55	55	55	55
α-Cellulose	63	57	43	62.4	56.4	42.4
Ax[g]	0	0	0	0.6	0.6	0.6
CHO[c]	0	6	20	0	6	20
Total	1000	1000	1000	1000	1000	1000

[b] Riken Vitamin, Tokyo, Japan.

[c] Nacalai Tesque, Kyoto, Japan.

[d] PUFA: (eicosapentaenoic acid) EPA 5 g and (docosahexenoic acid) DHA 5 g.

[e] Vitamin mix (12 g kg^{-1} diet) (Vitamin A free): *p*-aminobenzoic acid 0.092 g, biotin 0.004 g, inositol 3.668 g, nicotinic acid 0.368 g, Ca-pantothenate 0.552 g, pyridoxine-HCl 0.112 g, riboflavin 0.072 g, thiamine-HCl 0.036 g, menadione 0.036 g, α-tocopherol 0. 184 g,

cyanocobalamine 0.0008 g, calciferol 0.012 g, stay-C 1.36 g, folic acid 0.008 g, choline choride 5.5 g.

[f] Mineral mix (86 g kg^{-1} diet): K_2HPO_4 20.04 g, $Ca_3(PO_4)_2$ 27.39 g, $MaSO_4.7H_2O$ 30.62 g, $NaH_2PO_4.2H_2O$ 7.96 g.

[g] Ax: Carophyll Pink® containing 8% astaxanthin made by DSM Nutrition, Japan.

Table 4-2. Chemical analysis of the experimental diets.

Compositions	Ax_0			$Ax_{0.6}$		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}
Moisture (%)	12.36	11.25	13.11	12.76	12.29	11.33
Crude protein (%)	53.95	54.26	54.53	55.55	54.29	55.99
Crude lipid (%)	10	10.5	12.39	9.26	10.88	13.39
Ash (%)	7.22	7.35	7.34	7.55	7.53	7.81
Ax content (g kg^{-1})	ND[a]	ND	ND	0.54	0.55	0.54
CHO content (g kg^{-1})	0.2	5.8	19.1	0.1	6.1	18.6

[a] ND, not detected

Table 4-3. Growth parameters and nutrient utilization in kuruma shrimp fed with experimental diets for 56 days[a].

[a] Data were expressed as mean ± S.E.M. from triplicate groups.

Parameters	Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax} \times P_{CHO}$
IBW (g)	4.39±0.01	4.38±0.03	4.37±0.02	4.36±0.01	4.40±0.01	4.37±0.01	0.49	0.78	0.29
FBW (g)	6.34±0.02	6.77±0.17	5.99±0.26	6.79±0.26	7.03±0.26	6.75±0.23	0.08	0.02	0.51
BWG (%)	44.47±2.39	54.60±3.31	36.77±5.30	55.63±6.04	59.64±5.68	54.72±5.55	0.09	0.01	0.43
SGR (%)	0.66±0.05	0.78±0.07	0.56±0.12	0.79±0.12	0.83±0.11	0.78±0.11	0.09	0.01	0.39
FI (g)	27.45±0.55	26.90±0.55	29.24±0.62	27.45±0.55	26.35±0.18	28.70±1.17	0.01	0.51	0.89
FCR (%)	14.07±0.39	11.37±1.01	18.88±1.18	11.55±1.20	10.25±1.09	12.18±0.89	0.01	0.01	0.14
Survival (%)	81.48±3.70	85.19±6.42	70.37±5.85	81.48±3.70	88.89±11.11	74.07±12.25	0.01	0.39	0.82
HSI (%)	5.21±0.38	6.17±0.13	5.50±0.62	6.08±0.73	6.49±0.45	5.83±0.59	0.36	0.25	0.83

[b] Significant effects determined by two-way ANOVA.

Table 4-4. Whole body proximate analysis (% dry matter basis, except moisture) of kuruma shrimp fed with experimental diets for 56 days[a].

Parameters	Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax} \times P_{CHO}$
Crude protein (%)	67.39±0.6	70.11±1.33	66.91±0.51	66.81±0.66	67.92±0.93	70.17±0.85	0.54	0.91	0.32
Crude lipid (%)	3.96±0.24	4.04±0.43	4.42±0.13	3.82±1.05	4.12±0.27	4.39±0.03	0.07	0.23	0.59
Ash (%)	16.37±1.03	15.29±0.22	17.82±0.47	18.56±0.06	16.72±0.29	17.23±0.77	0.16	0.15	0.24

[a] Data were expressed as mean ± S.E.M. from triplicate groups.

[b] Significant effects determined by two-way ANOVA.

Table 4-5. Color parameters of cooked whole body and muscle of kuruma shrimp read by colorimeter which fed with experimental diets for 56 days[a].

Parameters		Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
		CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax}\times P_{CHO}$
Head	L^{*}[c]	59.83±0.46	60.88±1.14	59.19±1.07	61.12±0.20	59.32±0.08	58.94±0.48	0.62	0.89	0.63
	a^{*}	13.49±0.42^{a}	14.06±0.38^{a}	15.46±0.16^{b}	18.12±0.03^{c}	21.23±0.65^{e}	19.57±0.04^{d}	0.01	0.01	0.01
	b^{*}	24.65±0.66^{a}	26.49±1.33^{a}	29.54±0.30^{ab}	27.59±0.57^{a}	34.15±0.89^{b}	24.79±1.27^{a}	0.01	0.04	0.01
Body	L^{*}	62.55±1.34	63.34±0.06	63.15±0.98	62.08±0.14	61.00±0.47	60.90±0.79	0.94	0.04	0.45
	a^{*}	16.95±0.18^{a}	18.23±0.57^{ab}	18.64±0.45^{ab}	20.37±0.29^{bc}	22.09±0.57^{c}	19.61±0.03^{b}	0.02	0.01	0.02
	b^{*}	25.23±0.35^{a}	29.39±0.41^{ab}	29.14±0.32^{ab}	31.01±0.94^{bc}	34.62±0.87^{c}	28.54±0.04^{ab}	0.01	0.01	0.01
Muscle	L^{*}	66.21±0.70^{ab}	63.65±0.48^{ab}	66.40±0.97^{ab}	67.03±0.34^{b}	65.80±0.77^{ab}	62.19±1.23^{a}	0.05	0.54	0.01
	a^{*}	12.98±0.04^{a}	13.69±0.19^{a}	13.90±0.03^{a}	16.04±0.71^{b}	20.50±0.03^{d}	18.18±0.37^{c}	0.01	0.01	0.03
	b^{*}	19.30±0.82	26.93±1.54	22.63±0.65	22.70±1.71	27.53±1.37	26.93±1.14	0.01	0.04	0.37

[a] Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent significant difference from control group (P<0.05).

[b] Significant effects determined by two-way ANOVA.

[c] Lightness (L^{*}), yellowness (b^{*}) and redness (a^{*}) of kuruma shrimp head, body and muscle.

Table 6. Total astaxanthin (Ax) content of kuruma shrimp fed with experimental diets for 56 days[a]

Parameters		Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
		CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax}\times P_{CHO}$
Total Ax	Whole body	31.87±0.32^{a}	41.05±0.32^{b}	33.55±0.06^{a}	117.42±0.41^{cd}	119.93±0.03^{d}	115.5±0.98^{c}	0.01	0.01	0.01
	Carapace	60.11±0.35^{a}	60.69±0.50^{a}	68.56±0.72^{a}	157.47±1.90^{b}	203.3±3.48^{d}	187.85±2.28^{c}	0.01	0.01	0.01
	Muscle	30.94±0.08^{a}	35.72±0.26^{b}	34.56±0.56^{b}	68.33±0.47^{c}	82.58±0.59^{e}	77.74±0.16^{d}	0.01	0.01	0.01
	Head	47.19±0.11^{a}	47.76±0.22^{a}	60.97±0.12^{a}	107.43±0.16^{c}	127.11±0.03^{e}	113.39±0.72^{d}	0.01	0.01	0.01

[a] Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent significant difference from control group (P<0.05).

[b] Significant effects determined by two-way ANOVA.

Table 4-7. Digestive enzyme activity of kuruma shrimp fed with experimental diets for 56 days[a]

Parameters	Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax} \times P_{CHO}$
Protease	0.77±0.02	1.01±0.02	0.92±0.03	1.18±0.1	1.10±0.14	1.47±0.11	0.11	0.01	0.08
Lipase	0.01±0.01^{a}	0.09±0.04^{b}	0.11±0.01^{bc}	0.11±0.01^{bc}	0.10±0.01^{bc}	0.13±0.01^{c}	0.01	0.01	0.01
Amylase	0.09±0.02^{a}	0.12±0.01^{ab}	0.12±0.02^{ab}	0.14±0.02^{b}	0.15±0.01^{b}	0.13±0.01^{b}	0.06	0.01	0.07

[a] Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent significant difference from control group (P<0.05).

[b] Significant effects determined by two-way ANOVA.

Table 4-8. Neutral lipid (NL) of body content of kuruma shrimp fed with experimental diets for 56 days[a]

	Fatty acids[c]	Ax_0			$Ax_{0.6}$			Probability (P value)[b]		
		CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax}\times P_{CHO}$
NL	14:0	5.15±0.19	3.38±0.18	2.88±0.37	2.62±0.1	2.82±0.89	2.49±0.43	0.11	0.03	0.13
	15:0	0.44±0.07	0.22±0.01	0.33±0.01	0.31±0.09	0.34±0.01	0.45±0.11	0.24	0.49	0.14
	16:0	27.14±0.66	24.49±0.97	22.83±0.22	26.89±0.14	23.93±0.45	25.05±0.21	0.01	0.48	0.22
	17:0	0.83±0.17	0.71±0.28	0.56±0.05	0.92±0.05	0.99±1.12	0.82±0.02	0.42	0.12	0.78
	18:0	10.19±0.53	9.09±0.19	6.59±0.48	10.12±0.62	8.29±0.07	6.83±0.71	0.01	0.62	0.58
	15:1	0.21±0.02	0.09±0.04	0.23±0.01	0.15±0.01	0.18±0.05	0.14±0.01	0.29	0.35	0.04
	17:1	0.38±0.01	0.33±0.19	0.31±0.01	0.50±0.04	0.17±0.13	0.43±0.01	0.14	0.69	0.21
	18:1n-9	18.38±0.09	18.89±0.45	23.18±0.92	16.22±0.77	16.59±0.88	19.82±0.16	0.01	0.01	0.61
	20:1n-11	0.38±0.09	0.42±0.08	0.42±0.02	0.44±0.01	0.39±0.01	0.33±0.07	0.85	0.69	0.49
	20:1n-9	3.29±0.17	3.02±0.35	3.39±0.25	1.82±0.02	2.47±0.37	3.12±0.16	0.07	0.01	0.12
	22:1n-9	2.67 ± 0.22^{a}	1.91 ± 0.19^{a}	3.61 ± 0.08^{b}	2.69 ± 0.18^{a}	2.06 ± 0.03^{a}	2.29 ± 0.03^{a}	0.01	0.02	0.01
	16:2n-6	0.12 ± 0.06^{a}	0.95 ± 0.04^{b}	0.83 ± 0.01^{b}	1.03 ± 0.09^{b}	1.10 ± 0.09^{b}	0.94 ± 0.04^{b}	0.01	0.01	0.01
	18:2n-6	8.49 ± 0.13^{a}	13.36 ± 0.95^{bc}	14.25 ± 0.78^{c}	9.39 ± 0.38^{a}	9.69 ± 0.10^{ab}	15.15 ± 0.82^{c}	0.01	0.29	0.02
	18:3n-3	0.95±0.10	0.65±0.26	1.04±0.02	1.03±0.03	0.44±0.05	0.94±0.04	0.01	0.45	0.51
	20:3n-3	1.35±0.14	1.59±0.39	1.29±0.11	1.27±0.21	1.14±0.04	1.01±0.17	0.58	0.16	0.68
	20:4n-6	1.25±0.09	1.45±0.26	0.72±0.04	0.97±0.06	0.98±0.06	0.77±0.02	0.02	0.05	0.17
	20:5n-3	6.10±0.58	6.50±0.73	5.58±0.33	6.39±0.49	4.91±0.64	5.86±0.14	0.54	0.46	0.2
	22:5n-3	9.02±0.45	9.64±0.28	8.31±1.04	14.21±0.88	20.46±1.94	10.82±0.47	0.02	0.01	0.06
	22:6n-3	3.71±0.10	4.34±0.57	3.65±0.41	3.01±0.30	2.98±0.11	2.75±0.57	0.53	0.02	0.7
	∑SFA	43.76 ± 0.74^{d}	37.88 ± 0.29^{bc}	33.18 ± 0.52^{a}	40.87 ± 1.10^{cd}	36.37 ± 0.89^{ab}	35.64 ± 0.59^{ab}	0.01	0.32	0.03
	∑MUFA	25.29±0.46	24.64±0.94	31.13±0.14	21.83±0.54	21.76±1.23	26.12±0.12	0.01	0.01	0.35
	∑PUFA	30.95±0.29	37.46±1.19	35.65±0.58	37.3±0.54	41.69±1.94	38.24±0.56	0.01	0.01	0.25

[a] Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent significant difference from control group (P<0.05).

[b] Significant effects determined by two-way ANOVA.

[c] ∑SFA, saturated fatty acid; ∑MUFA, monounsaturated fatty acid; ∑PUFA, polyunsaturated fatty acid.

Table 4-9. Polar lipid (PL) of body content of kuruma shrimp fed with experimental diets for 56 days[a]

	Fatty acids[c]	Ax_0			$Ax_{0.6}$			Probability (*P* value)[b]		
		CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax}\times P_{CHO}$
PL	14:0	0.72±0.06	1.13±0.08	0.89±0.20	0.99±0.27	0.68±0.05	0.97±0.36	0.93	0.85	0.28
	15:0	0.52±0.09^{ab}	0.65±0.08^{ab}	0.82±0.02^{b}	0.81±0.09^{b}	0.64±0.02^{ab}	0.44±0.05^{a}	0.84	0.54	0.01
	16:0	35.72±0.41	33.89±0.13	34.3±1.68	31.51±1.3	28.19±0.71	27.84±0.21	0.05	0.01	0.51
	17:0	0.36±0.08	0.48±0.07	0.67±0.07	0.73±0.06	0.66±0.09	0.62±0.04	0.37	0.03	0.06
	18:0	27.27±0.46	25.02±0.3	22.99±0.38	26.59±1.02	25.28±0.47	25.57±1.71	0.11	0.44	0.35
	14:1n-5	0.41±0.02	0.41±0.04	0.42±0.01	0.43±0.04	0.39±0.02	0.51±0.03	0.08	0.19	0.17
	18:1n-9	9.19±0.24	9.31±0.65	11.27±0.54	9.28±1.18	11.56±0.57	13.16±0.75	0.02	0.05	0.33
	20:1n-11	0.80±0.03	0.85±0.05	0.82±0.03	0.81±0.01	0.89±0.01	0.92±0.07	0.24	0.15	0.53
	20:1n-9	0.56±0.16	0.68±0.07	0.92±0.04	1.24±0.16	0.83±0.05	1.23±0.11	0.49	0.01	0.08
	16:2n-6	1.12±0.02	1.37±0.16	1.11±0.08	1.09±0.05	1.09±0.05	1.09±1.04	0.33	0.18	0.32
	18:2n-6	0.54±0.09	0.42±0.05	0.41±0.06	0.64±0.02	0.38±0.01	0.51±0.18	0.18	0.46	0.66
	18:3n-3	2.18±0.03	1.38±0.15	1.38±0.12	2.55±0.36	1.71±0.17	1.17±0.09	0.01	0.31	0.28
	20:4n-6	6.39±0.09	4.19±0.44	4.17±0.73	6.09±0.51	4.49±0.12	5.76±0.41	0.01	0.19	0.17
	20:3n-3	0.615±0.03	1.52±0.24	0.91±0.02	0.45±0.23	1.06±0.16	1.13±0.01	0.01	0.3	0.16
	20:5n-3	1.32±0.08^{a}	1.31±0.07^{a}	1.84±0.04^{ab}	1.91±0.13^{ab}	2.13±0.27^{b}	1.58±0.06^{ab}	0.68	0.01	0.01
	22:4n-6	5.40±0.63	9.74±0.34	8.63±0.61	7.69±0.64	10.86±0.18	10.46±0.48	0.01	0.01	0.55
	22:5n-3	5.25±0.49	5.84±0.64	5.45±0.57	5.25±0.10	6.73±0.11	6.09±0.0.53	0.16	0.23	0.64
	22:6n-3	1.67±0.02	1.92±0.12	2.86±0.24	1.58±0.73	2.86±0.49	1.87±0.63	0.09	0.86	0.06
	SFA	64.58±0.18	61.15±0.22	59.67±0.92	60.62±1.02	55.46±0.73	55.42±0.04	0.01	0.01	0.64
	MUFA	10.96±0.86	11.24±0.55	13.43±1.12	11.75±0.35	13.67±1.01	15.81±0.48	0.02	0.03	0.53
	PUFA	24.46±0.67	27.69±0.39	26.76±0.65	27.24±0.28	31.28±0.42	29.62±0.61	0.01	0.01	0.71

[a] Data were expressed as mean ± S.E.M. from triplicate groups. Data with different superscript letters in one row represent significant difference from control group (P<0.05).

[b] Significant effects determined by two-way ANOVA.

[c] ∑SFA, saturated fatty acid; ∑MUFA, monounsaturated fatty acid; ∑PUFA, polyunsaturated fatty acid.

Table 4-10. Immune responses of kuruma shrimp fed with experimental diets for 56 days[a]

Parameters[c]	Ax_0			$Ax_{0.6}$			Probability (*P* value)[b]		
	CHO_0	CHO_6	CHO_{20}	CHO_0	CHO_6	CHO_{20}	P_{CHO}	P_{Ax}	$P_{Ax} \times P_{CHO}$
THC	21.5±2.5	30.0±1.0	29.0±1.0	38.5±3.5	63.0±2.0	67.5±2.5	0.34	0.01	0.23
PO	2.1±0.9	4.2±0.1	4.8±1.2	3.2±0.6	5.45±0.95	3.8±0.7	0.28	0.68	0.64
Lysozyme	0.36±0.01	0.44±0.01	0.39±0.01	0.44±0.01	0.48±0.01	0.41±0.04	0.07	0.04	0.43
MDA	8.81±0.92	4.58±0.39	4.96±1.69	2.65±0.77	2.46±0.58	3.11±1.01	0.36	0.03	0.33

[a] Data were expressed as mean ± S.E.M. from triplicate groups.

[b] Significant effects determined by two-way ANOVA.

[c] Parameters abbreviations: THC, the total haemocyte count; PO, phenoloxidase activity; MDA, malondialdehyde levels.

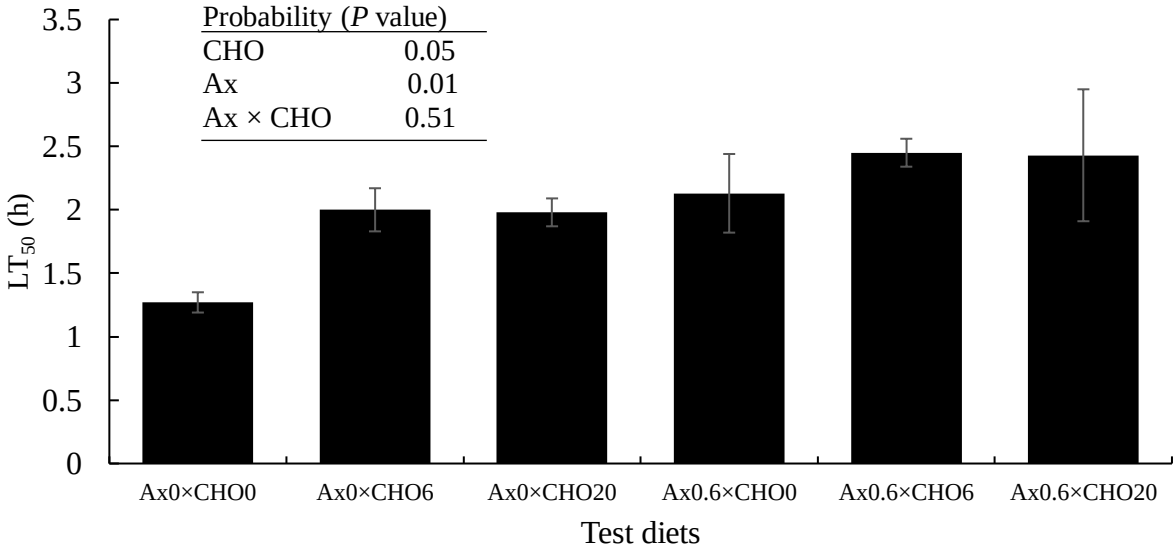

Fig. 4-1. Time to 50% mortality (LT_{50}) of kuruma shrimp exposed to low salinity water. Values are expressed as mean ± SEM from triplicate groups.

Chapter 5 : General Summary and Conclusions

The present study was carried out to investigate the dietary Ax effectiveness and determine the optimum dietary Ax level on different stages of kuruma shrimp, *Marsupenaeus japonicus*. Various growth, stress tolerance, immune response, and pigmentation parameters were employed to evaluate the influence of dietary Ax on the performance of kuruma shrimp. Interactive effects of dietary vitamin E and Ax, dietary cholesterol and Ax on pigmentation performance, tissue Ax content, tissue body composition, growth, immune response, and stress tolerance were also investigated. To summarize the present study results from different experiments, it was found that:

1. No positive correlation was detected between Ax content in test diets and survival rate of different stages of kuruma shrimp.
2. Excessive supplementation may be detrimental to both growth performance and stress resistance of kuruma shrimp.
3. Correctly dosed Ax supplementation was confirmed to give positive influence on larval stages of kuruma shrimp. A break-point at 168.9 mg kg^{-1} level Ax was estimated using the regression equation Y= 0.0015x + 5.824 (R^2 = 0.99) and Y = −0.0004x + 6.145 (R^2 = 0.68) on the total length measurement. A break-point at 82.1 mg kg^{-1} level Ax was estimated using the regression equation Y = 0.09x + 34.5 (R^2 = 0.95) and Y = −0.0262x + 44.043 (R^2 = 0.93) for formalin resistance.
4. Correctly dosed Ax supplementation was confirmed to give positive influence on post-larval stage of kuruma shrimp. A break-point at 108.7 mg kg^{-1} level Ax was estimated by the regression equation Y = 0.985x + 407.67 (R^2 = 0.98) and Y = −0.0447x + 516.7 (R^2 = 0.83) for body weight gain. A break-point at 178.1 mg kg^{-1} level Ax was estimated by the regression equation Y = 0.0083x + 1.818 (R^2 = 0.94) and Y = −0.0019x + 3.635 (R^2 = 0.7) for formalin resistance.
5. Correctly dosed Ax supplementation was confirmed to give positive influence on juvenile stage of kuruma shrimp. A break-point at 401 mg kg^{-1} diet level Ax was estimated using the regression equation Y = 0.0308x + 42.17 (R^2 = 0.96) and Y = -0.0047x + 56.42 (R^2 = 0.83) on the body weight gain measurement. A break-point at 420 mg kg^{-1} diet level Ax was estimated using the regression equation Y = 0.0108x + 5.055 (R^2 = 0.99) and Y = 0.0022x + 8.665 (R^2 =

0.97) on the total hemocyte count measurement. A break-point at 404 mg kg^{-1} diet level Ax was estimated using the regression equation Y = 0.009x + 11.055 (R^2 = 0.96) and Y = 0.0011x + 14.25 (R^2 = 0.79) on the redness (*a**) measurement of shrimp body.

6. Dietary Ax and α-Toc functioned interactively on growth performance and Ax content in kuruma shrimp.

7. Interactive effects by dietary CHO and Ax existed on growth parameters, lipase activity, pigmentation, and total Ax content in different parts of shrimp body.

Taken together, our results and those of previous investigations suggest that Ax is an effective nutritional supplement for larval kuruma shrimp, with the quantities suggested in this study serving as a good starting point for further investigation into Ax metabolism and physiology. We suggest optimized dietary levels for larvae of 168.9 mg kg^{-1} for growth and 82.1 mg kg^{-1} for stress tolerance performance. For post-larvae, optimal levels are 108.7 mg kg^{-1} for growth and 178.1 mg kg^{-1} for stress tolerance performance. For juvenile, optimal levels of Ax are around 400 mg kg^{-1} for better growth performance and stress resistance responses. Notably, excessive supplementation may lead to the paradoxical effects on the growth performance and stress resistance for kuruma shrimp. Therefore, we suggested using care in supplementing diets for kuruma shrimp. Dietary *α*-Toc may help to reduce the Ax consumption, and increase deposition of Ax in the muscle. Furthermore, the addition of CHO can enhance the positive effects of dietary Ax. These enhanced effects were possibly due to the increasing levels of Ax found in whole body, muscle, carapace, and head tissues.

In conclusion, our results demonstrate the potential economic benefits of Ax supplementation in kuruma shrimp aquaculture systems, through improved growth performance, and enhanced pigmentation and immune responses. Our study also fills a data gap regarding the interactive effects of dietary factors and provides practical information to improve pigmentation and performance of kuruma shrimp, thus enhancing their commercial value.

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