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Effect of dietary astaxanthin on growth and immune response of Giant freshwater prawn *Macrobrachium rosenbergii* (de man)

VIJAY KUMAR, BINDU R. PILLAI*, P. K. SAHOO, J. MOHANTY and S. MOHANTY

Central Institute of Freshwater Aquaculture
Kausalyaganga, Bhubaneswar - 751002, Orissa, India

Abstract

Effects of dietary incorporation of astaxanthin on growth and selected immune parameters of freshwater prawn *Macrobrachium rosenbergii* were examined. Sub-adult *M. rosenbergii* were fed diets fortified with astaxanthin at 0 (control), 25, 50, 100 and 200 mg kg^{-1} for a period of 28 days. Results revealed a significant increase ($P<0.05$) in specific growth rate of prawns fed 50, 100 and 200 mg kg^{-1} astaxanthin enriched diet compared to the control diet. Among the immune parameters evaluated, phenoloxidase activity and total protein showed a significant increase ($P<0.05$) in prawns fed diets enriched with 50, 100 and 200 mg kg^{-1} astaxanthin. The total haemocyte count was also significantly higher in prawns fed diets enriched with 50 and 100 and 200 mg kg^{-1} astaxanthin. Total carotenoid content in muscle, exoskeleton and hepatopancreatic tissues showed a dose dependent increase. The present results showed that dietary incorporation of astaxanthin at 50, 100 and 200 mg kg^{-1} level had significant positive effects on growth and immune response of *M. rosenbergii.*

Introduction

The giant freshwater prawn (*Macrobrachium rosenbergii*, Palaemonidae) has become the most important cultured freshwater prawn species in many tropical and sub tropical countries due to its superior cultivable attributes such as fast growth rate, large size, hardiness, euryhaline nature and compatibility with carps. It also has consumer preference, and domestic as well as export market demand (New and Valenti, 2000). The global aquaculture production of this species has shown a quantum jump from 19,035 metric tonnes in 1995 to 194,159 metric tonnes in 2004 (FAO, 2006).

In many aquaculture operations, feed contributes to more than 60% of production costs. Hence, it is essential to look for feed ingredients that improve growth rate and

*Corresponding Address: Tel: +91-674-2465421
Email address : bindupillaicifa@gmail.com

survival of the cultured species to reduce the production cost. Carotenoid pigments are used as key nutritional additives in several fish and crustacean diets due to their beneficial properties. Carotenoids are a family of over 600 natural lipid soluble pigments that are produced by algae (including phytoplankton), higher plants and photosynthetic bacteria (Meyers and Latscha, 1997).

Astaxanthin (3,3-dihydroxy-4,4-diketo-β,β-carotene) is a red carotenoid pigment occurring naturally in a wide variety of living organisms and is the major carotenoid of crustaceans, comprising about 90% of the total pigment in *Marsupenaeus japonicus* (Ishikawa et al., 1966). Since crustaceans, like other aquatic animals, are unable to produce astaxanthin *de novo*, it must be available either in their native habitat or manufactured diet to meet nutritional requirements. Improved pigmentation and survival due to dietary astaxanthin supplementation has been reported in kuruma prawn, *M. japonicus* and tiger prawn, *Penaeus monodon* (Chien and Jeng, 1992; Thongrod et al., 1995; Chien et al., 1999). Dietary astaxanthin has also been reported to enhance antioxidant defense capability in *P. monodon* juveniles and consequently provide protection to some extent against *Vibrio damsela* challenge in the early stage (Pan et al., 2001).

The majority of studies on the effects of dietary incorporation of astaxanthin were concentrated on salmon and penaeid shrimps. The present work was undertaken to study the effect of dietary astaxanthin on growth and immune parameters of the freshwater prawn, *M. rosenbergii*.

Materials And Methods

Live prawns for the experiment were collected from the freshwater prawn farm of the Central Institute of Freshwater Aquaculture, Bhubaneswar, Orissa. India. The prawns were acclimated for one-week prior to the experiment. Test diets were prepared by adding different levels of astaxanthin i.e. 25 mg kg^{-1} (A_{25}), 50 mg kg^{-1} (A_{50}), 100 mg kg^{-1} (A_{100}), and 200 mg kg^{-1} (A_{200}) to the control diet (A_0). The composition of the control or basal diet is provided in Table 1. Astaxanthin ($C_{40}H_{52}O_{4;}$ MW = 596,86) was procured from ACROS Organics, Belgium and stored at 4°C until use. The feed ingredients were thoroughly mixed, manually pelletized, air dried in a dark room and stored at -20° C. Experiments were conducted in ferro-cement tanks (3 m x 1.2 m x 1 m). Three replicate tanks were set up for each treatment. After one-week of acclimation, prawns were individually measured for length and wet weight and those in intermoult stage were selected for experimentation. Ten prawns were released into each tank.

Water temperature was measured twice daily at 0600 and 1400 h. Dissolved

The prawns were provided with the test diets at 5% of the body weight twice daily in two divided doses at 0800 and 1700 h throughout the experimental period. The duration of theexperiment was 28 days.

Table 1. Composition of control (basal) feed.

Ingredients	Composition (%)
Ground nut oil cake	60
Fish meal	10
Prawn meal	10
Wheat flour	8
Rice bran	8
Vitamin and Mineral Mix*	2
Vegetable Oil (ml/kg)	2

* Each 250 g Vitamin and Miner al Mix provides vitamin A (5,000,000 IU), vitamin D_3 (100,000 IU), vitamin B_2 (0.2 g), vitamin E (75 units), vitamin K (0.1 g), calcium pantothenate (0.25g), nicoten amide (1.0 g), vitamin $B_{12,}$(0.6 mg), choline chloride 15 g, calcium (75 g), manganese (2.75 g), iodine (0.1 g), iron (0.75 g), zinc (1.5 g), copper (0.2g) and cobalt (0.045 g).

oxygen, pH and ammonia levels of water were measured once every week following standard procedures (APHA, 1998). Prawns were monitored daily for moulting and mortality. At the end of the feeding trial, length and weight of each prawn in each of the experimental group were recorded and weight gain and specific growth rate of the experimental and control groups were calculated. Specific growth rate was obtained using the formula:

Specific growth rate (SGR) = $(\ln W_f - \ln W_i) \times 100 / t$

Where, $\ln W_f$ = the natural logarithm of the final weight,

$\ln W_i$ = the natural logarithm of the initial weight, and

t = time (number of days) between $\ln W_f$ and $\ln W_{i.}$

Total carotenoid content in different tissues including exoskeleton, hepatopancreas and abdominal muscle of the experimental prawns was measured spectrophotometrically following Arredondo-Figueroa et al. (2003). The carotenoid was extracted using acetone and the total carotenoid concentration (TCC) was calculated from the peak absorbance in acetone at 460 nm.

At the end of the experiment haemolymph was collected from pericardial sinus

of three prawns from each tank using a 26-gauge needle and 1 ml syringe containing anticoagulant solution (1:9) (tri-sodium citrate: 0.114 M, sodium chloride: 0.1M, pH 7.45) to measure the phenoloxidase activity (PO) activity following Hernandez-Lopez et al. (1996). Haemocyte lysate supernatant (HLS) was prepared from the collected haemolymph by homogenizing the PBS-washed haemocyte suspension in a sonicator equipped with a micro tip and centrifuging (12,000 x g) the same. The PO activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA, Hi Media, Mumbai) as substrate in U-bottomed microwell plates. HLS (50 µl) was pre-incubated for 15 min at 37°C, after which 100 µl of L-DOPA (1.6 mg/ml) in tris buffer saline (TBS: 50 mM Tris, 100 mM NaCl, pH 7.3) was added and allowed to react for 1 min. Each reaction mixture was further diluted with 100 µl TBS and absorbance was measured at 490 nm. The control solution, which consisted of 50 µl of PBS (to replace HLS), 100 µl L-DOPA, and 100 µl TBS, was used for the background phenoloxidase activity in all test conditions. The PO activity in terms of optical density was expressed as dopachrome formation/50 µl HLS. For total haemocyte count, 100 µl haemolymph was drawn from another three prawns from each tank using 1 ml sterile syringe (26-gauge needle) containing 900 µl anticoagulant (sodium chloride 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 20 mM, pH 4.5) with fixative solution (sodium cacodylate 0.10 M and 1.5% glutaraldehyde, pH 7.8) in 1:1 ratio. A drop of haemolymph was placed on a haemocytometer to measure total haemocyte count (THC) using a phase contrast microscope.

To measure total protein in the sera, haemolymph was collected from rest of the animals as described above without anticoagulant and allowed to clot at room temperature for 30 min and left at 4°C for 1hr. The tubes were then centrifuged at 5000 x g at 4°C for 30 min to collect the supernatant serum. Total protein in the sera was estimated following Bradford (1976) using bovine serum albumin as the standard protein.

Data were analyzed using one-way analysis of variance. Means were compared using Duncanís multiple range test (Duncan, 1955). Significance was tested at $P<0.05$ levels.

Results and Discussion

The mean water quality parameters during the study period: water temperature 26±2°C, pH 7.8±0.06, dissolved oxygen 6.8±0.2 mg l^{-1}, ammonia nitrogen 0.03±0.006 mg l^{-1} were well within the optimum range reported for the species. No significant differences ($P>0.05$) were observed in the water quality parameters between different treatments.

The prawns were in moult stage C (intermoult) at the beginning of the experiment.

Overall the numbers of moults were higher in prawns fed 50, 100 and 200 mg kg^{-1} astaxanthin enriched diets. The mean weight gain was minimum in control prawns and maximum in prawns fed 200 mg kg^{-1} astaxanthin enriched diet (Table 2).

The specific growth rate was significantly higher ($P<0.05$) in prawns fed with

Table 2. Growth and survival of sub-adult *Macrobrachium rosenbergii* fed diets containing different levels of astaxanthin for 28 days. Each value represents mean ± SD.

Parameters	Treatment				
	A_0 (Control)	A_{25}	A_{50}	A_{100}	A_{200}
Initial weight (g)	16.1 ± 2.8	17.8 ± 2.9	18.5 ± 3.0	15.3 ± 2.8	18.5 ± 3.1
Final weight (g)	17.1 ± 2.9	18.9 ± 3.0	20.6 ± 3.2	17.4 ± 2.9	20.8 ± 3.1
Weight gain (g)	$1.00 \pm 0.10_a$	$1.10 \pm 0.19_a$	$2.10 \pm 0.22_b$	$2.10 \pm 0.19_b$	$2.30 \pm 0.21_b$
SGR (%)	$0.22 \pm 0.03_a$	$0.21 \pm 0.02_a$	$0.38 \pm 0.02_b$	$0.46 \pm 0.03_b$	$0.42 \pm 0.01_b$
Survival (%)	100	100	100	100	100

* Mean values bearing different superscript in a row differ significantly ($P < 0.05$)

50, 100 and 200 mg kg^{-1} astaxanthin enriched diet. Growth rate of prawns fed with 25 mg kg^{-1} astaxanthin enriched diet was not significantly different ($P>0.05$) from control prawns (Table 2). Previous studies on astaxanthin supplementation in penaeid shrimps also reported similar results. *M. japonicus* fed with 50 and 100 mg kg^{-1} of astaxanthin supplemented diet showed increase in growth (Chien and Jeng, 1992). When fed diets supplemented with astaxanthin at levels of 0, 5, 15, 60, and 300 mg kg^{-1} for 30 days, growth of post-larvae of *P. monodon* increased significantly as dietary astaxanthin levels increased (Thongrod et al., 1995). Dietary astaxanthin improved the growth rate and shortened the moulting cycle of *M. japonicus* post larvae during a 20 day rearing (Petit et al.1997). Arredondo-Figueroa et al. (2003) while studying the effect of dietary astaxanthin on growth of the shrimp *Litopenaeus vannamei,* suggested that astaxanthin influences the physiological functions and enhances the nutrient assimilation in shrimps thereby enhancing the growth rate.

No mortality was recorded in any of the treatment groups including control; hence it is difficult to conclude on the effect of astaxanthin on survival of *M. rosenbergii*. However, several studies with penaeids have reported increased survival in astaxanthin fed groups (Yamada et al. 1990; Chien and Jeng, 1992; Negre-Sadargues et al. 1993; Pan et al. 2001).

Total carotenoid content in exoskeleton, hepatopancreas and muscle tissue of prawns fed with 50, 100 and 200 mg kg^{-1} of astaxanthin-enriched diet was significantly higher than that of control prawns (Figure 1). Among the tissues studied, total carotenoid content is highest in the hepatopancreas, followed by exoskeleton and muscle tissue. Tissue carotenoid concentration was found to increase with an increase in the concentration of astaxanthin in the diet. Previous studies on astaxanthin supplementation and tissue carotenoid concentration in decapod crustaceans also reported similar results (Yamada et al. 1990; Chien and Jeng, 1992; Menasveta et al. 1993; Harpaz et al. 1998).

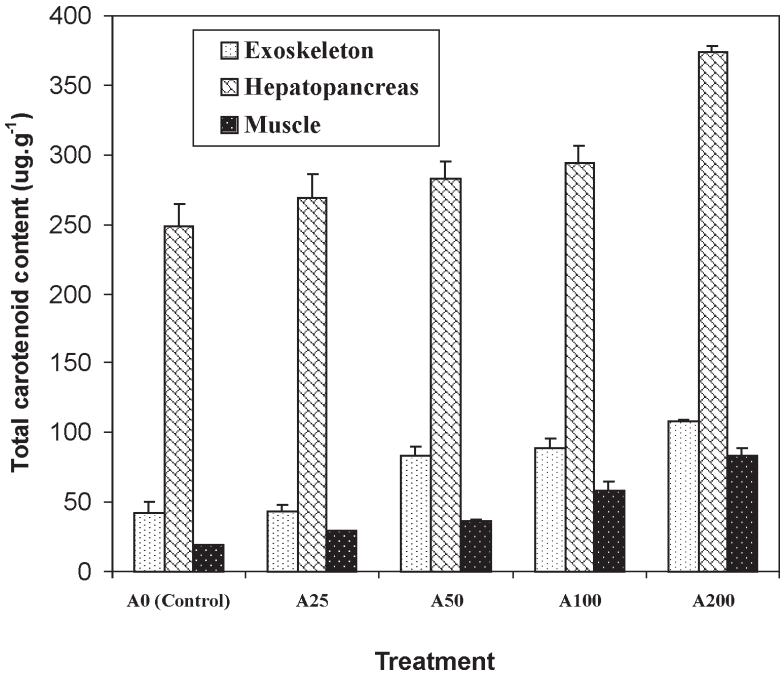

Figure 1. Total carotenoid concentration in exoskeleton, hepatopancreas and abdominal muscle tissue of *Macrobrachium rosenbergii* fed diets containing different levels of astaxanthin for 2 8 days. Each bar represents mean ± SD.

Phenoloxidase (PO) activity was significantly higher ($P<0.05$) in prawns fed astaxanthin enriched diets compared to control prawns (Table 3).

A dose dependent increase in PO activity could be observed up to 100 mg kg^{-1} of astaxanthin incorporation. PO activity has been employed as one of the important immune parameters to study the status of immune system, immunomodulation and disease resistance in crustaceans (Smith et al., 2003). An enhancement in PO activity is thought to enhance the immune ability of animals (Cheng et al., 2004, Baruah and Pani Prasad, 2001). Enhanced PO activity observed in astaxanthin fed groups in the present study may thus indicate enhanced immune capability of these prawns. Astaxanthin has been reported to improve both specific and non-specific immune response mechanisms in

Table 3. Phenoloxidase activity, total haemocyte count (THC) and serum total protein content of *Macrobrachium rosenbergii* fed diets containing different levels of astaxanthin for 28 days. Each value represents mean ± SD.

Groups	Phenoloxidase activity	THC (10^7 cells ml^{-1})	Total protein (g dl^{-1})
A_0 Control	0.339 ± 0.03[a]	0.97 ± 0.09[d]	16.04 ± 0.72[f]
A_{25}	0.503 ± 0.06[b]	0.85 ± 0.07[d]	16.34 ± 1.2[f]
A_{50}	0.605 ± 0.06[b]	1.46 ± 0.12[e]	18.10 ± 1.67[g]
A_{100}	0.804 ± 0.08[c]	1.30 ± 0.45[e]	21.52 ± 0.35[h]
A_{200}	0.620 ± 0.08[b]	1.12 ± 0.96[e]	20.15 ± 0.82[h]

*Mean values bearing different superscript in a column differ significantly ($P < 0.05$).

decapod crustaceans. In tiger prawn *P. monodon,* 100 to 200 ppm dietary astaxanthin has been shown to improve resistance to bacterial and viral infection (Menasveta, 1995). Dietary astaxanthin has also been reported to enhance the antioxidant defense capability in *P. monodon* juvenile and provide protection against *Vibrio damsela* challenge (Pan et al., 2003)

A significant increase ($P<0.05$) in total serum protein content could be observed in groups fed with 50, 100 and 200 mg kg^{-1} astaxanthin compared to control (Table 3). However, total serum protein content of prawns fed 25 mg kg^{-1} astaxanthin did not vary significantly ($P>0.05$) from the control group. The increase in protein content may be due to the increase in defense molecules in prawns fed with higher doses of astaxanthin, and may indicate a better immune status in those animals. Several immune molecules have been identified and purified in crustaceans such as the lipopolysacchride-binding protein, b- glucan binding protein and peptidoglycan binding protein (Vargas-Albores et al., 1993; Hall et al., 1995). Exposure to any of the immunostimulatory substances is expected to raise one or more of these defense proteins in particular and the total protein levels in the haemolymph in general.

The total haemocyte count obtained in the control group prawns was 0.97 x 10^7 cells ml^{-1} of haemolymph. THC showed a significant increase in prawns fed with 50, 100 and 200 mg kg^{-1} of astaxanthin for 28 days (Table 3). Blood cells or haemocytes in arthropods play an important role in defense mechanisms against parasites and pathogenic or non-pathogenic microorganisms that enter the blood stream through

wounds in the cuticle (Johansson and Soderhall, 1989). In crustaceans an increase in THC has been related to disease resistance (Rodriguez and Moullac, 2000). Previous studies in decapod crustaceans have found that dietary astaxanthin improves non-specific immune response mechanisms (Pan et al., 2003). The higher THC is reported to provide an enhanced immune capability during periods of higher activity or enhanced environmental bacterial loads (Moullac and Haffner, 2000; Chand et al., 2006). The increase in the number of circulating haemocytes recorded in the present study might be due to the higher haemocyte mobilization indicating a better immune status in that group.

The present study revealed that dietary incorporation of astaxanthin at 50, 100 and 200 mg kg^{-1} level significantly enhanced the growth and immune response of sub-adults of M. rosenbergii. The incorporation of astaxanthin at 50 mg kg^{-1} was sufficient to enhance the growth of prawns as there was no significant difference in growth among prawns fed with 50, 100 and 200 mg kg^{-1} astaxanthin enriched diet. Dietary astaxanthin was deposited in exoskeleton, hepatopancreas and abdominal muscle tissue of prawns.

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