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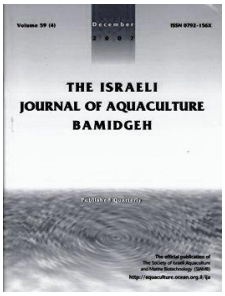


# Carotenoids in Integument, Muscle, and Midgut Gland of Red Shrimp *Pleoticus muelleri* Bate, 1888 (Crustacea, Penaeoidea) Fed Carotenoid-Supplemented Diets

**A.C. Díaz[1]*, S.M. Velurtas[1], A.V. Fernández Gimenez[2], S.N. Mendiara[1], J.L. Fenucci[2]**

[1] *Comisión de Investigaciones Científicas Provincia de Buenos Aires (CIC), Departamento de Ciencias Marinas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina*

[2] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Funes 3350, B7602AYL, Mar del Plata, Argentina*

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**Abstract**

Carotenoids stimulate immune systems in animals. Since animals cannot synthesize carotenoids, they must be included in feeds. Oxidative pathways suggested for the metabolism of dietary carotenoids include β-carotene and astaxanthin. The objective of this study was to compare growth, survival, and the carotenoid profile in the integument, muscle, and midgut gland of juvenile red shrimp (*Pleoticus muelleri*) fed isoproteic formulated feeds containing astaxanthin or β-carotene. Juveniles (5.15±0.941 g) were fed one of four diets containing 50 or 100 mg/kg of the carotenoid. The control group was fed a diet without carotenoid supplementation. A spectroscopy UV/visible method produced no evidence supporting a possible influence of these pigments on growth or survival. However, there were significant statistical differences in carotenoids in the integument (carapace and epidermis) and muscle between animals fed the different diets. The integument had the highest carotenoid concentrations: 14.91±4.064 µg β-carotene, 7.47±1.252 µg free astaxanthin, and 18.31±5.40 µg esterified astaxanthin per gram tissue (avg of five treatments). Only β-carotene (1.74±0.161 µg/g tissue) was stored in the muscle. We conclude that, due to the high cost of artificial pigments, dietary carotenoid supplementation is not necessary for grow-out.

* Corresponding author. E-mail: acdiaz@mdp.edu.ar

## Introduction

The Argentine red shrimp *Pleoticus muelleri* is an open thelycum species, distributed along South American coastal waters from Rio de Janeiro, Brazil (23°S), to Santa Cruz, Argentina (49°45'S). Males can reach 50 g and females 90 g total weight; in some cases, spawning exceeds 360,000 eggs per female (Díaz and Fenucci, 2004). The Aquaculture Group from the University of Mar del Plata has been working with *P. muelleri* on different aspects of its biology, nutrition, maturation, large-scale larvae culture, and pond grow-out (Fenucci, 2004).

Understanding the nutritional requirements of a species is essential to ensuring profitable production and long-term sustainability in aquaculture. The market value of shrimp is predominately based on the visual appeal of their color. Under culture conditions, crustaceans are limited to artificial diets that might lack important bioactive metabolites. The use of bioactive substances such as nutritional additives to improve cultured shrimp yields is receiving increased attention and there are efforts to define the biological function of carotenoids as a dietary supplement.

Carotenoids play an important role in human health by acting as biological antioxidants that protect cells and tissues from the damaging effects of free radicals (Halliwell and Gutteridge, 2001). In crustaceans, carotenoids stimulate the immune system, increase stress tolerance, serve as a source of vitamin A, and enhance embryonic development (Liñán-Cabello et al., 2002). Commercially, they are used as food colorants and in nutritional supplements.

The concentration of carotenoids in crustaceans is affected by environmental factors such as diet (i.e., carotenoid content), the color of the background substrate, light intensity, photoperiod, temperature, and other factors (Rao, 1985). Shell color in decapod crustaceans is primarily determined by the carotenoid astaxanthin, which is incorporated into a macromolecular protein complex known as crustacyanin (Chayen et al., 2003).

Since crustaceans are unable to synthesize carotenoids *de novo*, astaxanthin or appropriate precursors must be supplied in the diet (Meyers and Latscha, 1997). The enhancement of pigmentation can be attributed to the type, composition, and concentration of pigments contained in the diet (Yamada et al., 1990), digestibility of the material itself (Chien and Jeng, 1992), and possibly the presence of cofactors in the material involved in absorption and deposition. Astaxanthin is the predominant carotenoid in penaeoids. It is present as free and esterified (monoester and diester) astaxanthin, or bound to protein as a carotenoprotein (Pan and Chien, 2003). Crustaceans can transform carotenoids into a limited range of closely related derivatives; oxidative pathways suggested for the metabolism of dietary carotenoids include β-carotene and zeaxanthin to astaxanthin (Yamada et al., 1990).

The objectives of the present study were to compare the growth and survival of juvenile *P. muelleri* fed isoproteic formulated feeds containing different levels of carotenoids, and to determine the carotenoid profile in the integument, muscle, and midgut gland.

## Materials and Methods

*Experimental conditions*. Animals were obtained from the coastal waters of Mar del Plata, Argentina (38°05'S 57°W). Feeding trials were carried out on juveniles (5.15±0.941 g initial wt) held in 150-l glass aquaria containing undergravel filters with sand and a crushed shell bed. Experimental conditions were maintained constant at a salinity of 33‰, pH 7, temperature 18±1°C and a photoperiod of 11 h light:13 h dark. Shrimp were stocked at a density of 20/m². Feed intake, mortality, and the presence of exuviae were recorded daily. Individual shrimp weights were determined at the beginning of the experiment and after 50 days. At the end of the experiment, animals from each treatment were anesthetized on ice and pooled for analysis of growth and whole body composition. Growth performance and survival were measured in terms of final individual weight, percent weight gain = 100(final mean wt - initial mean wt)/initial mean wt, and percent survival.

*Treatments*. Treatments consisted of four feeds supplemented with one of two carotenoids: astaxanthin or β-carotene, each at two dietary carotenoid concentrations, 50 or 100 mg/kg diet, plus a control group fed a formulated diet without carotenoid supplementation. Formulations were made according to the contents of the protein sources to obtain isoproteic and isolipidic feeds (Table 1). The chemical compositions of the diets were confirmed through proximate analysis according to AOAC (1997). All ingredients were mixed, cold pelleted (<50°C) by extrusion to obtain 3-mm diameter pellets, oven-dried at 50°C for 24 h, air-dried, flushed with argon gas, and stored in darkness at -4°C.

Table 1. Formulation and proximate analysis of the control diet.

| *Ingredient (g/100 g)* | *%* |
|---|---|
| Fishmeal (65% crude protein) | 48.0 |
| Soybean meal (42% crude protein) | 17.0 |
| Corn starch | 20.0 |
| Wheat | 9.5 |
| Fish oil | 2.0 |
| Fish soluble | 2.0 |
| Soybean lecithin | 0.5 |
| Cholesterol | 0.5 |
| Vitamins* | 0.5 |
| *Proximate analysis* | *%* |
| Crude protein | 39.3 |
| Crude lipid | 7.3 |
| Ash | 1.1 |
| Moisture | 5.9 |

* g/kg: cholecalciferol 1.8, thiamin 8.2, riboflavin 7.8, pyridoxine 10.7, calcium panthothenate 12.5, biotin 12.5, niacin 25.0, folic acid 1.3, B12 HCl 1.0, ascorbic acid (Rovimix Stay C) 39.1, menadione 1.7, inositol 0.3, choline chloride 0.2, α-tocopherol acetate 75, vitamin A acetate 5

During the first week of rearing, shrimp were fed the control diet to stabilize their body pigment. A random sample of 10 shrimp was collected for initial body carotenoid analysis. Feeds were tested in three replicate groups of eight shrimps, each, for seven weeks. The animals were fed *ad libitum* once a day. The feeding rate was adjusted daily in each tank to minimize feed waste.

*Carotenoid analysis*. After one week (initial) and seven weeks, shrimp were dissected into integument (carapace, telson, uropod), muscle, and midgut gland. The dissected parts were freeze-dried, liophilized samples were homogenized in an argon atmosphere in darkness, and carotenoids were analyzed following a modification of the technique proposed by Schiedt et al. (1993). β-carotene was extracted three times with hexane in an argon atmosphere in darkness. Free astaxanthin was separated by partitioning with dimethyl sulphoxide (DMSO)/acetone (1:3) until colorless in an inert atmosphere. Esterified astaxanthin was extracted according to the technique of Napoli and Horst (1989), which consists of saponification with KOH in ethanol and extraction with hexane; the samples were evaporated in a nitrogen atmosphere and resuspended in chloroform.

Absorption peaks of the carotenoids were identified by scanning the spectrum between 200 and 750 nm using a diode array spectrophotometer (Shimadzu UV-2102 PC, UV/Visible Scanning Spectrophotometer). Carotenoid concentrations were calculated using standard curves of β-carotene in hexane ($1.88 \times 10^{-6}$ M) and astaxanthin in DMSO/acetone ($4.19 \times 10^{-6}$ M) using the specific extinction coefficients 122,000 and 124,000 M-1/cm, respectively (Perkampus, 1992).

*Statistical analysis*. One-way analysis of variance (ANOVA) was used to test the significance of the overall treatment effects on weight gain, survival rate, and tissue carotenoid content. Data were expressed as means±standard error. Pearson's rank correlation coefficient was used to identify significant correlations among carotenoid contents, weight gains, and survival. In all cases, differences were significant when $p<0.05$ (Sokal and Rohlf, 1995).

## Results

After seven weeks of rearing, there were no significant differences between treatments in percent weight gain or survival (Table 2). Neither β-carotene nor astaxanthin was detected in the midgut gland. β-carotene was detected in the integument and muscle (Table 3). There were significant statistical differences between the initial and final β-carotene concentrations in the integument (avg of five treatments, 14.91±4.064 µg/g tissue) and muscle (avg of five treatments, 1.74±0.161 µg/g tissue). Esterified

Table 2. Weight gain and survival of juvenile red shrimp (*Pleoticus muelleri*) fed formulated feeds containing one of two carotenoids at one of two dietary concentrations (mean±SE; n = 3).

| | *Mean wt (g)* | | Mean wt | *Survival* |
|---|---|---|---|---|
| *Diet* | *Initial* | *Final* | gain (%) | *(%)* |
| Control | 5.21±0.114 | 6.87±0.528 | 31.7 | 41.7 |
| 50 β-carotene | 5.23±0.076 | 7.17±0.383 | 37.0 | 58.0 |
| 100 β-carotene | 5.28±0.074 | 7.51±0.340 | 42.3 | 66.3 |
| 50 astaxanthin | 5.25±0.239 | 6.99±0.098 | 33.3 | 58.3 |
| 100 astaxanthin | 5.26±0.122 | 6.23±0.902 | 18.3 | 54.0 |

astaxanthin was detected only in the integument and was significantly higher at the end of the experiment (avg of five treatments, 18.31±5.40 µg/g tissue) than at the beginning. Free astaxanthin was also detected only in the integument (avg of five treatments, 7.47±1.252 µg) and was significantly lower than the initial value in all treatments except the control. Carotenoid concentrations, calculated from absorption spectra, are shown in Fig. 1.

Table 3. Initial and final body carotenoids (µg/g tissue) of juvenile red shrimp *Pleoticus muelleri* fed formulated feeds containing one of two carotenoids at one of two dietary concentrations (mean±SE; n = 3).

| | | *Diet* | | | |
|---|---|---|---|---|---|
| | *Initial* | *Control* | *50 β-carotene* | *100 β-carotene* | *50 astaxanthin* | *100 astaxanthin* |
| *B-carotene* | | | | | | |
| Integument | 3.94±1.520$^{a}$ | 19.40±3.262$^{b}$ | 11.72±1.378$^{b}$ | 11.04±2.790$^{b}$ | 13.29±1.147$^{b}$ | 19.10±7.276$^{b}$ |
| Muscle | 0.91±0.154$^{c}$ | 2.22±0.464$^{d}$ | 1.67±0.166$^{d}$ | 1.03±0.035$^{c}$ | 1.38±0.233$^{d}$ | 2.42±0.654$^{d}$ |
| *Esterified astaxanthin* | | | | | | |
| Integument | 11.89±1.515$^{e}$ | 26.70±4.054$^{f}$ | 15.53±1.046$^{f}$ | 13.28±2.300$^{f}$ | 15.45±0.332$^{f}$ | 20.59±6.026$^{f}$ |
| Muscle | ND | ND | ND | ND | ND | ND |
| *Free astaxanthin* | | | | | | |
| Integument | 10.67±1.119$^{g}$ | 9.07±2.303$^{g}$ | 7.61±1.114$^{h}$ | 6.77±1.687$^{h}$ | 6.75±0.197$^{h}$ | 8.76±0.686$^{h}$ |
| Muscle | ND | ND | ND | ND | ND | ND |

Means in a row with different superscripts significantly differ ($p$<0.05)
ND = not detected

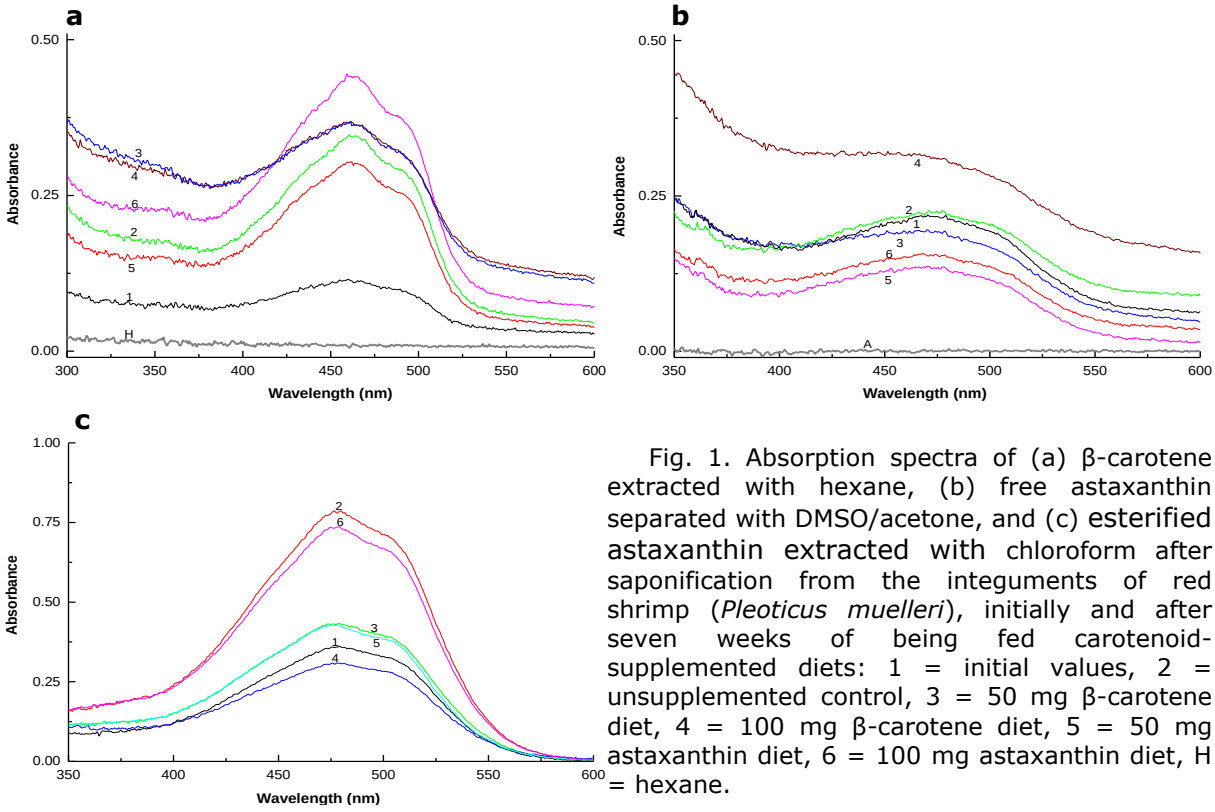


Fig. 1. Absorption spectra of (a) β-carotene extracted with hexane, (b) free astaxanthin separated with DMSO/acetone, and (c) esterified astaxanthin extracted with chloroform after saponification from the integuments of red shrimp (*Pleoticus muelleri*), initially and after seven weeks of being fed carotenoid-supplemented diets: 1 = initial values, 2 = unsupplemented control, 3 = 50 mg β-carotene diet, 4 = 100 mg β-carotene diet, 5 = 50 mg astaxanthin diet, 6 = 100 mg astaxanthin diet, H = hexane.

## Discussion

There was no evidence supporting a possible influence of β-carotene or astaxanthin on growth or survival. Analogous results have been reported for other penaeoid species fed carotenoid-supplemented diets (Yamada et al., 1990; Chien and Jeng, 1992; Nègre-Sadargues et al., 1993; Boonyaratpalin et al., 2001). Regardless of the concentration of the supplemented carotenoid, survival of crustaceans was unaffected (Chien and Shiau, 2005). Further research is necessary to test the influence of carotenoids on growth and survival of shrimp cultivated under stress conditions.

The required level of dietary carotenoid to maintain body astaxanthin varies with the target tissue. Astaxanthin deposition in the shell (190%) was more sensitive than in the flesh (90%) in *Marsupenaeus japonicus* fed diets containing 50 or 100 mg/kg astaxanthin (Chien and Shiau, 2005). In *Penaeus monodon*, body astaxanthin decreased despite dietary supplementation with astaxanthin at 50 mg/kg (Menasveta et al., 1993) or 80 mg/kg (Pan et al., 2001).

Even when carotenoids are supplemented in the diet, the initial body pigment can be hard to maintain. In *Litopenaus vannamei*, the concentration of total carotenoid is higher in wild shrimp than in captive shrimp, probably because of the greater concentration and availability of carotenoids that usually occurs in the natural environment of shrimp (Liñán-Cabello et al., 2003). However, in the present work, the initial carotenoid levels were lower than those in the shrimp after seven weeks of rearing under controlled conditions. In most cases, the carotenoid tissue concentrations of the shrimp fed the supplemented diets did not significantly differ from those in shrimp fed the control. The same pattern was observed in *P. monodon* under experimental conditions, although the carotenoid content in shrimp fed a diet supplemented with carotenoid (astaxanthin or β-carotene) was much greater (two or four fold) than that of shrimp fed an unsupplemented control (Boonyaratpalin et al., 2001). Our results indicate that the control diet is an efficient and inexpensive source of carotenoids for pigmentary purposes in *P. muelleri*. The dietary carotenoids were dissolved in marine- derived oil, which may yield an additional benefit by assisting in maintaining the integrity of the chemical compounds during extended periods of storage.

Pigmentation of crustaceans may be achieved more efficiently when biosynthetic intermediates that are structurally close to the stored forms of the pigments are provided in the diet (Shahidi et al., 1998). The main carotenoids in penaeoid exoskeletons are astaxanthin, astaxanthin esters, and β-carotene (Nègre-Sadargues et al., 1993; Meyers, 1994; Pan and Chien, 2003). Carotenoids lead to the deposition of mainly astaxanthin esters in the integument (carapace and epidermis), but the time required for each conversion (step) has never been established or documented (Chien and Shiau, 2005). In the present study, there were higher relative amounts of esterified astaxanthin in the epidermis in all treatments, demonstrating the biotransformation of both β-carotene and astaxanthin. Similar results were obtained in *M. japonicus* fed synthetic carotenoids (Nègre-Sadargues et al., 1993).

Our results suggest that astaxanthin esterification may be necessary for the incorporation of unesterified astaxanthin into the carotenoid binding protein complex and eventual deposition in the shell. Esterified astaxanthin is present in the epithelial tissues of crustaceans where it may have a storage function (Dall, 1995; Sagi et al., 1995; Pètit et al., 1998). As a highly lipophilic molecule, astaxanthin can readily be stored in animals in lipid globules only as an astaxanthin ester, which is more water soluble, or within carotenoprotein complexes (Wade et al., 2005).

In the present work, carotenoids were not detected in the midgut gland. In contrast, there was a significant increase in total carotenoids in the midgut gland of mature female *L. vannamei* (Liñán-Cabello et al., 2003). As in most crustaceans, ovaries and midgut glands contain a complex mixture of carotenoid pigments, the relative proportions of which change markedly during ovarian maturation (Liñán-Cabello et al., 2002). Dietary carotenoids may enhance reproductive capacity and could represent the ability of carotenoids to bind vitellin into a lipoglyco-carotene protein complex, by which means

the macromolecule accumulates in the oocyte cytoplasm as a source of food for the embryo.

The use of natural pigments in feed formulations offers advantages over the use of synthetic alternatives (Sun et al., 1995). The use of synthetic pigments in feeds requires that the pigment be stabilized in a convenient form, and soluble in water or fat. There is considerable variation in carotenoid contents not only between species but also between geographic regions (Yanar et al., 2004). The design of inexpensive high quality diets is extremely important as feed costs represent more than 50% of the production costs for most aquaculture enterprises. It is relevant to identify whether the diet used in culture has a level sufficient to maintain normal pigmentation. In this study, there were no significant differences between treatments in carotenoid tissue concentrations and the initial level was lower than the final levels in all groups. These results indicate that the control diet is efficient for pigmentary purposes in this species. Further research is necessary to test the influence of carotenoids on the growth and survival of shrimp cultivated under stress conditions.

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