Effect of Dietary Astaxanthin on Free Radical Scavenging Capacity and Nitrite Stress Tolerance of Postlarvae Shrimp, *Pleoticus muelleri*

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ABSTRACT: The aim of this study was to investigate the effect of astaxanthin feed supplementation and environmental nitrite stress in postlarvae of *Pleoticus muelleri* (15 ± 0.004 mg initial weight) under culture conditions. Diets containing three levels of astaxanthin, 0 mg kg^{-1} of diet (C_0), 100 mg kg^{-1} of diet (C_{100}), and 300 mg kg^{-1} of diet (C_{300}), were used. Postlarvae fed with each diet were exposed to different concentrations of nitrite (NO_2Na) (0−200 mg L^{-1}). The 96 h median lethal concentration (LC_{50}) values of nitrite N were 76.3, 89.7, and 157 mg L^{-1} for shrimps fed to C_0, C_{100}, and C_{300}. The scavenging properties were evaluated against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by electron resonance spectroscopy (EPR). For all feed treatments, the extracts exhibited strong DPPH scavenging activity; however, shrimp fed with C_{100} and C_{300} showed the greatest activity to quench DPPH (62 and 59%, respectively) with respect to C_0 (43%). It can be concluded that astaxanthin acts as a protector of nitrite stress in *P. muelleri*.

KEYWORDS: *Pleoticus muelleri*, astaxanthin, scavenging capacity, nitrite, histopathology

INTRODUCTION

In recent years, products from various sources have proliferated as additives for aquaculture, which provide greater resistance to stress and enhance growth. These compounds are a very heterogeneous family considering their origin, chemical nature, and biological activity. Pigments have received a special interest because they produce benefits on growth and survival.[1] Carotenoids are among the most common natural pigments and are responsible for many of the red, orange, and yellow hues in plant leaves, fruits, and flowers, as well as the color of some birds, insects, fish, and crustaceans.[2] A high intake of carotenoids through foods is considered beneficial for human health because of their often assigned antioxidant activity. They are located in the hydrophobic core of the bilayer of the plasma membrane and other cellular membrane components, such as the microsome and vacuole. Polarity of carotenoids regulates membrane fluidity by generating a hydrophobic barrier in the membrane to polar molecules and ions.[3]

In crustacean penaeoids, astaxanthin is the least reducing carotenoid and the predominant pigment, representing 86−98% of total carotenoids.[4] Penaeoid shrimp cannot synthesize carotenes *de novo*, and consequently, these must be obtained through diet. Several sources of astaxanthin have been found and identified in many microorganisms, including the micro-algae *Haematococcus pluvialis*, *Chlorella zofingiensis*, and *Chlorococcus* sp., the red yeast *Phaffia rhodozyma*, and the marine bacterium *Agrobacterium aurantiacum*.[5]

In fish, astaxanthin is a precursor of vitamin A.[6] On the other hand, it improves embryonic and larval development as well as promotes cellular protection from photodynamic damage.[7] It has been demonstrated to be of equal importance to α-tocopherol as an antioxidant and raises environmental stress tolerance in *Penaeus monodon*.[8] Astaxanthin has an especially high propensity to absorb the excess energy from singlet

oxygen, releasing it as heat and returning oxygen and itself back to the ground state. This neutralization of singlet oxygen is known as quenching. Extracellular antioxidant defenses are measured through the so-called total antioxidant capacity, which is considered the cumulative action of all antioxidants present in body fluids, being an integrated parameter rather than a simple sum of measured antioxidants.[9−11] Under normal physiological conditions, there is a balance between the generation of free radicals and antioxidant protection; however, there is an increase in the oxidative stress when tissue damage occurs. Free radical scavenging activity of astaxanthin can be evaluated by spectroscopy and electron paramagnetic spin resonance (EPR) spectroscopy methods. EPR is considered to be the most accurate method for the detection of free radicals. The radical scavenging ability is determined by the disappearance of 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, with a distinctive EPR signal. DPPH reaction with antioxidants can be followed by loss of the EPR signal or loss of absorbance at 540 nm.[12]

The midgut gland plays an important role in the absorption of dietary carotenoids and is also a sensitive indicator of nutritional status and diseases in various shrimp species.[13−15] Furthermore, this organ has been considered as the site of carotenoid storage and the pathway of pigment transfer into the hemolymph.[16] Histological analysis of the midgut gland has been used as a practical means to assess the nutritional condition in the shrimp culture.[17,18]

In crustaceans, high levels of nitrite affect health and survival of individuals. Previous studies on *Pleoticus muelleri* have never

examined the histological changes of this organ related to toxicity of nitrite. Nitrite is an intermediate compound in bacterial nitrification and denitrification processes; therefore, an imbalance in these processes can lead to a built up of nitrite in aquatic habitats.[19]

Red shrimp *P. muelleri* has a large distribution area in southwest Atlantic waters from 20° S, Espíritu Santo, Brazil, to 50° S, Santa Cruz, Argentina. This shrimp has acquired great interest on account of its potential use in culture in temperate areas; therefore, it is important to determine the optimal culture conditions to have healthy individuals.

The aim of this research was to investigate the interaction between the astaxanthin concentration in the feed and antioxidant response to exposure to nitrite in postlarvae of *P. muelleri*. For this purpose, the effects of these compounds on growth, survival, free radical scavenging, and functional morphology of the midgut gland were evaluated.

■ MATERIALS AND METHODS

Chemicals. The DPPH standard was obtained from Aldrich. Astaxanthin (98%) was obtained from Sigma and stored in the dark at 253 K. Sodium nitrite ($NaNO_2$) and all other reagents were analytical-grade. The solvents were from Merck, and they were carefully purified following the literature method to have them free of alkenes and carbonyl compounds. All solvents used were dried conveniently.

Experimental Animals. Specimens of *P. muelleri* were reared from hatchery-raised postlarvae (15 ± 0.004 mg initial weight) at Nágera Station, Marine Science Department, Mar del Plata National University, Mar del Plata, Argentina. Feeds were tested in three replicate groups of 90 individuals each for 30 days. Shrimps were stocked at a density of 10 per 0.1 m^2 in aquaria with continuous aeration, and filtered seawater (to 5 μm) was exchanged at a rate of 50% per day. The experimental conditions were as follows: photoperiod, 12 h light/12 h dark; temperature, 20 ± 0.8 °C; salinity, 33 g L^{-1}; and pH, 7.

The treatments consisted of three feeds (37% protein and 7.5% lipids) supplemented with astaxanthin at concentrations of 0 mg of astaxanthin kg^{-1} of diet (C_0), 100 mg of astaxanthin kg^{-1} of diet (C_{100}), and 300 mg of astaxanthin kg^{-1} of diet (C_{300}). Feeds were freshly prepared every 2 weeks to avoid carotenoid decomposition during storage (at −20 °C). Measurements confirmed the stability of the carotenoids within this period of time by scanning the spectrum between 200 and 750 nm using an ultraviolet–visible (UV–vis) spectrophotometer. Formulations of feed were made according to the chemical composition results of the byproduct meal to obtain isoproteic and isolipidic feeds.[20] All ingredients, from a local feed manufacturer, were mixed and cold-pelleted (<50 °C) by extrusion[21] and were oven-dried for 24 h at 50 °C (Table 1).

All groups of animals were fed *ad libitum* once a day. Upon completion of the feeding trial, surviving postlarvae from each treatment were weighed and transferred to glass jars with four postlarvae of each in triplicate for the static test of acute toxicity nitrite (NO_2Na). Nitrite test solutions were prepared with seawater at concentrations of 0, 10, 20, 40, 80, 100, 120, 160, and 200 mg L^{-1}. Animals were not fed during experimental trial. Shrimp behavior and death were monitored at 24 h intervals up to 96 h.

At the end of the static test, some postlarvae from each treatment and control individuals were fixed in Davidson fluid for 24 h and processed using routine techniques for light microscopy.[22] The fixed tissues were dehydrated in ascending concentrations of ethyl alcohol and embedded in paraffin. The tissue sections were cut to ∼5 μm and stained with hematoxylin and eosin (H&E).

EPR Sample Preparation. About 25 mg of lyophilized tissue of postlarvae from each treatment and control individuals was mixed with 1 mL of chloroform under an argon atmosphere. Each reaction mixture contained 50 μL of the tissue solution and 50 μL of DPPH (2.8×10^{-5} M) solution. Control or blank preparations, containing 50 μL of DPPH in chloroform solvent, remained unaltered after a long

Table 1. Percent Composition of the Control Feed

ingredients (g 100 g^{-1})	C_0
fish meal (65% crude protein)[a]	48.0
soybean meal (42% crude protein)[b]	17.0
corn starch	20.0
squid protein concentrate	1.0
wheat bran	8.5
fish oil	2.0
fish soluble	2.0
soybean lecithin	0.5
cholesterol	0.5
vitamin supplement[c]	0.5

[a]Agustinier S.A., Mar del Plata, Argentina. [b]Melrico S.A., Buenos Aires, Argentina. [c]Cholecalciferol, 1.8 g/kg; thiamin, 8.2 g/kg; riboflavin, 7.8 g/kg; pyridoxine, 10.7 g/kg; calcium panthothenate, 12.5 g/kg; biotin, 12.5 g/kg; niacin, 25.0 g/kg; folic acid, 1.3 g/kg; B_{12} HCl, 1.0 g/kg; ascorbic acid (Rovimix Stay C), 39.1 g/kg; menadione, 1.7 g/kg; inositol, 0.3 g/kg; choline chloride, 0.2 g/kg; α-tocopherol acetate, 75 g/kg; and vitamin A acetate, 5.0 g/kg.

period of time. Samples for EPR measurements were prepared in quartz tubes with an internal diameter of 4 mm to achieve an adequate tuning at a constant room temperature of 293 K and were analyzed at fixed time intervals for 60 min. DPPH proved to be a very stable free radical;[9] the intensity of the signal decays with time and represents the radical–tissue reaction.

EPR Measurements. Potential antioxidant activity of postlarvae extracts was determined on the basis of the scavenging activity of the stable free radical DPPH using EPR with a Bruker ELEXSYS E 500 T spectrometer, operating at X-band. Amplitude signals were transformed in radical concentrations by comparing the area under the EPR absorption spectrum of the sample to that of a chloroform solution of the concentration standard, DPPH. All data were recorded at the same acquisition parameters: microwave power, 12.93 mW; microwave frequency, 9.71 GHz; modulation frequency, 100 kHz; attenuation, 12 dB; modulation amplitude, 100 mT; magnetic field center, 346.5 mT; sweep width, 10 mT; and sweep time, 60 s for three scans.[25] Measurements were repeated at least 3 times to minimize random errors. The standard plotting program Origin Pro was used to fit all sets of points whenever necessary.

Statistical Analysis. The analysis of variance and least significant difference tests were conducted to identify differences among means. Data were reported as the mean ± standard deviation. The concentration of nitrite lethal to 50% of the test organisms in a specified time period (LC_{50}) values were derived from regression of Probit-transformed mortalities against the log concentration[23] with the computer software XLSTAT 2010. The estimated Probit line and the results of a χ^2 test for goodness of fit were computed. In all cases, significance was set as $p < 0.05$.[24]

■ RESULTS AND DISCUSSION

After 30 days of rearing trial with feeds C_0, C_{100}, and C_{300}, percent weight gain ranged from 172 to 190%, with no significant differences between treatments. In most cases, there is no positive effect observed on growth of crustacean penaeoids fed with carotenoid-supplemented feeds.[26,27] Some studies showed that dietary astaxanthin has a positive effect on shrimp survival. In the study by Niu et al.,[28] growth performance and survival in *Litopenaeus vannamei* were significantly higher when fed diets with supplemental astaxanthin (100−400 mg kg^{-1}) related to shrimp fed with the control diet and there were no significant differences among the three dietary treatments supplemented with astaxanthin. However, in *Marsupenaeus japonicus*, similar survival between shrimp fed at different concentrations of pigmented feeds was

observed, indicating that carotenoid supplemented at 100 mg kg^{-1} had no extra benefit on the survival of shrimp when compared to 50 mg kg^{-1}.[27]

In *P. muelleri*, survival was between 71 and 82%, with no significant differences among them, even with feeds supplemented with astaxanthin; the use of carotenoids as a nutritional additive would not be necessary for this species if they are fed diets containing animal meals, such as fish or squid, or vitamin A as a supplement.

The LC$_{50}$ values of nitrite N (mg L^{-1}) at 96 h estimated for shrimps by the Probit statistical method were 76.3, 89.7, and 157 mg L^{-1} fed with C$_0$, C$_{100}$, and C$_{300}$, respectively (Table 2).

Table 2. LC$_{50}$ of Nitrite to *P. muelleri* Fed with Experimental Feeds after 96 h of Exposure

feed	LC$_{50}$ (mg L^{-1})	confidence intervals (95%)		χ^2
		low limit	upper limit	
C$_0$	76.3	51.1	61.5	0.28
C$_{100}$	89.7	13.1	55.4	0.46
C$_{300}$	157	9.4	57.8	0.51

Research in fish and other aquatic animals have disclosed that nitrite is a disrupter of multiple physiological functions, including ion balance, respiratory, cardiovascular, endocrine, and excretory processes.[19] Enhancement of resistance to environmental stress in penaeoid shrimp postlarvae was associated with an increase in dietary and body astaxanthin.[8] Present results showed that astaxanthin improves shrimp health in terms of resistance against nitrite stress; the median lethal concentration of nitrite increases with higher astaxanthin dietary concentrations. In the trial testing nitrite N tolerance, it was determined that the 96 h LC$_{50}$ values for shrimps fed with C$_0$, C$_{100}$, and C$_{300}$ were significantly lower than the results reported by Lin and Chen[29] for *L. vannamei* juveniles (322 mg L^{-1}) but higher than those found for freshwater prawn *Macrobrachium rosenbergii*, with values between 8.8 and 12.9 mg L^{-1}. When the toxicological effects of nitrite in aquatic ecosystems are checked, it can be concluded that seawater animals are more tolerant to nitrite toxicity than freshwater animals, probably because of the improving effect of chloride ions on the tolerance of aquatic animals.[30]

Figure 1 shows the histology of the midgut gland of *P. muelleri*. Control treatment (no addition of nitrite) shows normal morphology of the midgut gland, composed of many tubules lined by a simple epithelium with clearly differentiated cells, namely, embryonic (E), fibrillar (F), absortive (R), and blister-like (B). Individuals of all nitrite treatments showed tissular alterations compared to the initial histological conditions. The main histopathological alteration was the retraction of the epithelium within the layer of laminar connective tissue that surrounds each tubule. Some tubules had a reduction in the number and size of store lipid vacuoles, while other tubules were completely devoid of epithelial cells and with tubular lumen dilated. Both types of tubules contained sloughed epithelial cells and cellular debris. Histological structure analysis of shrimps from treatment groups with elevated nitrite levels displayed varying degrees of tissue pathology in the midgut gland. It was observed in the sample of animals fed with astaxanthin and submitted at the highest concentration of nitrite that the general structure of the organ studied was less affected than the sample of those fed C$_0$. This allows for the inference that the addition of astaxanthin in feed
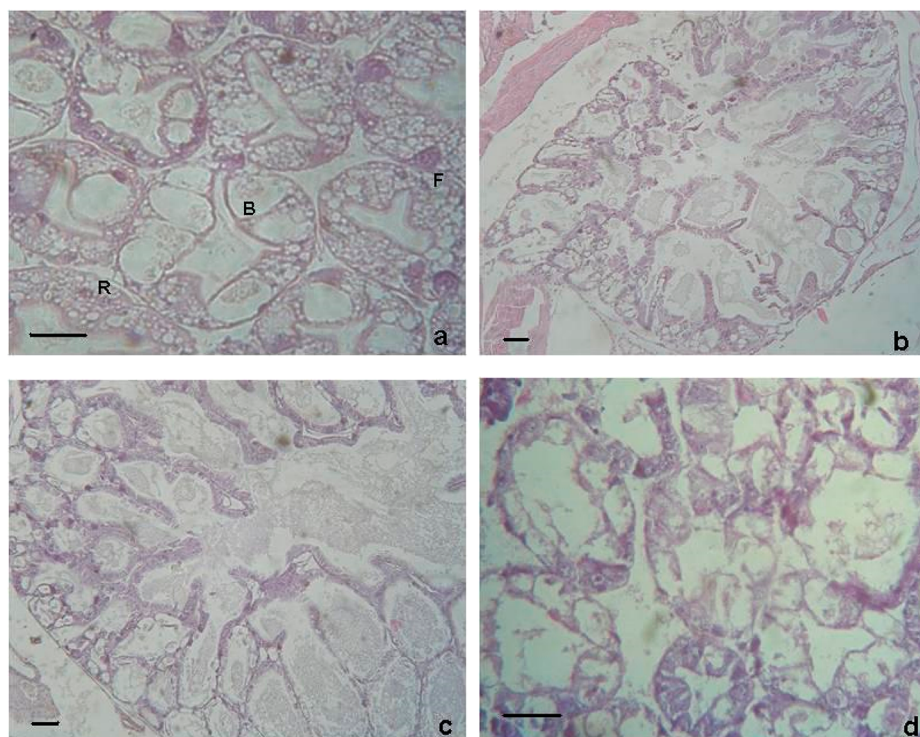

Figure 1. Transverse section through the midgut gland from postlarvae *P. muelleri* stained with H&E: (a) control treatment (no addition of nitrites) showing normal tubules, with B, B cell; F, F cell; and R, R cell, with a scale bar of 50 μm; (b) treatment with C$_{300}$ and 80 mg/L nitrite, with a scale bar of 200 μm; (c) treatment C$_0$ with 10 mg/L nitrite, with a scale bar of 100 μm; and (d) treatment C$_0$ with 80 mg/L nitrite, with a scale bar of 50 μm.

avoids the damage in the animals subjected to concentrations of 80 mg L^{-1} nitrite. All of these changes suggest that elevated nitrite levels negatively affect the health of the shrimp even if outward signs are no evident, such as growth and survival. According to Vogt,[31] the process of desquamation in the midgut gland of *P. monodon* starts with the cell lysis, particularly R cells, and the neighboring cells protrude in small basolateral extensions, pushing the damaged cells into the tubular lumen, generating ulcerations. In the present work, degenerative desquamation of the tubular epithelium in the midgut gland of *P. muelleri* was a pathological feature observed with all nitrite concentrations tested. This alteration was also observed in marine crustaceans acclimatized to low salinities, such as *Artemesia longinaris*[32] and *P. muelleri*,[33] and freshwater prawn *Palaemonetes argentinus* under hypersaline conditions.[34] The increased desquamation produced by different stressors resulted in a high rate of cell loss that does not make possible the restitution of the damage tissue. The midgut gland plays an important role in lipid metabolism; alterations observed in R cells suggest that the shrimp not metabolizing feeds normally might be due to the enhanced energy demand in response to stress by high concentrations of nitrite.[35]

In Figure 2, the kinetics of the DPPH reaction of tissue homogenates of shrimp fed with C_0 and C_{300} exposed at different sublethal concentrations of nitrite (0, 20, 40, and 80 mg L^{-1}) can be observed. Figure 2a (C_0) shows that free radical scavenging activity was detected at all tested nitrite concentrations; the signal decayed between 34 and 52% at 30 min. Similar kinetic curves were observed for control and 20 mg L^{-1} nitrite, and the lowest activity was at 80 mg L^{-1} nitrite. Higher contents of carotenoids in the feed (300 mg kg^{-1}) promoted production of protective substances, thereby increasing the quenching free radical in the system (Figure 2b). There exist some mechanistic explanations for the radical scavenging reactions of carotenoids *in vitro*,[36] but it is difficult to be certain which antioxidant role that carotenoids play in biological systems and the possible interference of toxic stressors, such as nitrite. The extracts of individuals exposed at 80 mg L^{-1} nitrite concentration for all feed treatments showed DPPH scavenging capacity, although some differences were noted among shrimp fed with C_0 (43%) and those fed with C_{100} and C_{300}, which had the greatest activity to quench DPPH (62 and 59%, respectively) (Figure 3).

Carotenoids have been reported to be the most efficient quenchers of singlet oxygen ($^1O_2$). Astaxanthin as a member of the carotenoid family reduces the oxidation rate;[37] for this reason, it plays an important role in animal health as an antioxidant through inactivation of the free radical produced from normal cellular activity and various stressors.[38] A previous work on *P. muelleri* demonstrated that the postlarval stages (with higher carotenoid concentrations) promoted a greater percentage of decay of DPPH over time, because radicals are consumed in the tissue at a speed that depends upon the amount of protective substances.[25] The long-standing controversy of function of carotenoids as antioxidants may be related to extrapolation of properties determined in homogeneous solution to more complex biological systems, where other factors, such as spatial organization and interaction between antioxidants, become important.[39] Nitrite exerts part of its toxicity through the formation of reactive oxygen intermediates (ROIs) that are able to attack almost all biomolecules present in their vicinity, causing protein modification, lipid peroxidation, and DNA damage. Activities
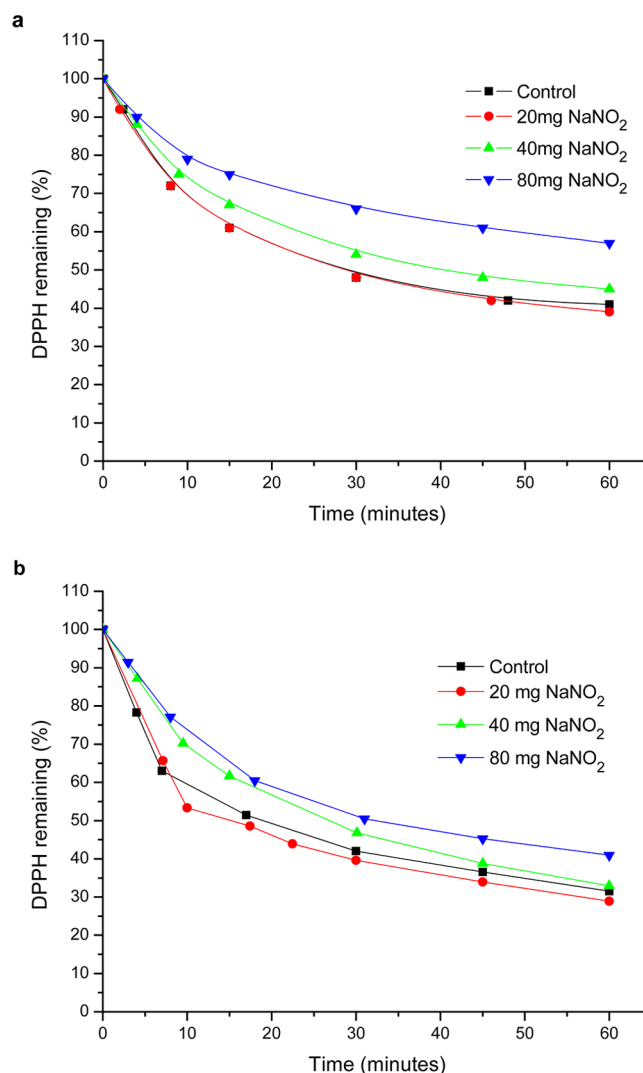

Figure 2. Reaction kinetics of tissue with the DPPH radical. Tissue extracts were tested for a pool of animals exposed at different concentrations of nitrite, by mixing 0.025 g of tissue with 50 μL of DPPH solution (2.8×10^{-5} M). All tests were conducted in triplicate, and the means are used: (a) animals fed with C_0 (without astaxanthin) and (b) animals fed with C_{300}.

of the three major antioxidant enzymes (glutathione peroxidase, superoxide dismutase, and catalase) responsible for combating the effects of ROIs diminished significantly in response to levels of ambient nitrite in *L. vannamei*.[40] Polar carotenoids, such as astaxanthin, are localized perpendicularly in the plasma membrane, consequently, allowing for the protection of the unsaturated lipids from the adverse effects of the oxidants.[41]

Results of the present work show a relationship between the carotenoid feed supplementation and the protective capacity of the homogenate against the nitrite concentration. Chien et al.[8] suggest that astaxanthin is a "semi-essential" nutrient for *P. monodon*, mainly when the animal is under physiological stress. Because an enhancement of antioxidant capacity by dietary astaxanthin in *P. muelleri* was observed. One of the most important aspects of this study is to optimize culture techniques for this species. The use of bioactive substances, such as nutritional additives, to improve performance in shrimp farming is receiving increasing attention, and efforts are being made to define the role of astaxanthin as a dietary supplement. The
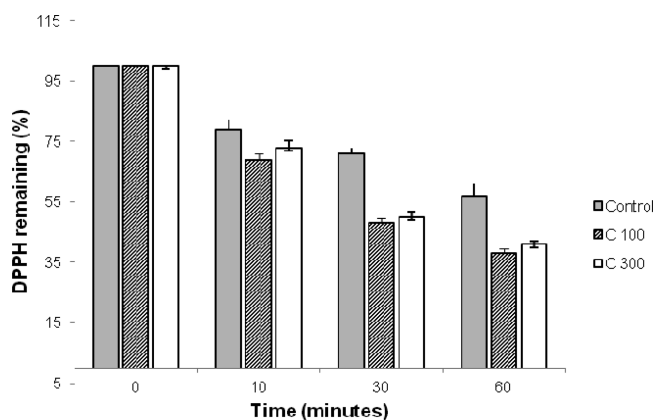
Figure 3. DPPH scavenging effect followed up to 60 min. Midgut gland extracts for a pool of animals exposed at 80 mg of nitrite and fed with different diets were tested by mixing 0.025 g of tissue with 100 μL of DPPH solution (2.8×10^{-5} M). Vertical bars represent the standard deviation of each data point ($n = 3$).

addition of carotenoid pigments as bioactive additives, which affect physiological and molecular processes under controlled conditions, is more evident as a result of not be synthesized *de novo*. It can be concluded that these compounds act as protectors of ambient nitrite under culture conditions. Further studies are necessary to identify the free radicals that contribute to total antioxidant activities of carotenoids under different physiological conditions and to determine the relationship between their radical scavenging properties and their biological effects.

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Notes
The authors declare no competing financial interest.

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