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Ana C. Díaz [a b], Susana M. Velurtas [a], Sara N. Mendiara [a] & Jorge L. Fenucci [a c]

[a] Departamento de Ciencias Marinas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3350, B7602AYL, Mar del Plata, Argentina

[b] CIC, La Plata, Argentina

[c] CONICET, Buenos Aires, Argentina

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Correlation between radical scavenging capacity and carotenoid profile during *Pleoticus muelleri* larval development

Ana C. Díaz[ab]*, Susana M. Velurtas[a], Sara N. Mendiara[a] and Jorge L. Fenucci[ac]

[a]*Departamento de Ciencias Marinas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3350, B7602AYL, Mar del Plata, Argentina;* [b]*CIC, La Plata, Argentina;* [c]*CONICET, Buenos Aires, Argentina*

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The purpose of this study was to measure the concentration of carotenoids and properties which occur in tissues to neutralize free radicals during ontogeny of *Pleoticus muelleri*. The stages of nauplius, protozoea, mysis and postlarvae of 1, 6, 10, 26, 30 days were examined from hatchery raised postlarvae from wild females. The β-carotene and the astaxanthin from the lyophilized tissue were quantified using a ultraviolet–visible spectrophotometer. Free radicals scavenging properties of tissues extracts were evaluated by electron paramagnetic resonance (EPR). The reaction of protective substances was followed with the 1,1-diphenyl-2-picryhydrazyl (DPPH) radical. The concentration of carotenoids of the whole larvae and postlarvae ranged from 1.72 to 87.04 $\mu g\, g^{-1}$ for β-carotene, and 2.26 to 19.86 $\mu g\, g^{-1}$ for astaxanthin. All the larval and postlarvae stages showed a protective capacity. In the stages of mysis, postlarvae 1 and 30 the homogenates were monitored without DPPH. The undefined EPR signal was quantified and considered as a ''pool'' of persistent radicals, with a concentration about 10^{-5} M. A relationship was observed between the concentration of carotenoids and the protective capacity of the homogenate. The postlarvae stages had a high concentration of carotenoids and the greatest protective capacity.

Keywords: Crustacea; Penaeoidea; scavenging capacity; DPPH; carotenoid; larval rearing

Introduction

Biological systems interact with the external factors to maintain an internal environment that enhances survival, growth and reproduction. The organisms began the evolutionary process of evolving antioxidant defence systems to protect against the free radicals toxicity; these systems produce endogenous antioxidant and oxidative enzymes (Halliwell and Gutteridge 2001). To prevent the damage of tissues, it is important to neutralize free radicals; however, the internal production of antioxidants is insufficient to neutralize and scavenge them completely. Electron paramagnetic resonance (EPR) is a highly suitable and potent tool for measuring the level of oxidative stress in vitro and in vivo (Díaz et al. 2004; Buico et al. 2008). The antioxidant activity of a molecule is measured by evaluating its ability to scavenge radicals; in this study the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was employed.

Living organisms have a series of antioxidant systems that counteract the generation of free radicals. Antioxidant defence mechanisms are formed by enzymatic components (e.g. superoxide dismutase, catalase, cytochrome oxidase and other peroxidases) and non-enzymatic such as vitamin E and β-carotene (as free radical scavengers in membranes), ascorbic acid, uric acid and glutathione (for the aqueous phase) (Winston et al. 1998). Thus, having a variety of antioxidants provides protection against various reactive oxygen species (ROS) in different compartments of the cell. Under normal physiological conditions, these defence mechanisms maintain a low concentration of ROS and free radicals in the cell; the balance between production and antioxidant defences determines the degree of oxidative stress (Valavanidis et al. 2006). Extracellular antioxidant defences are measured through the so-called total antioxidant capacity which is considered the cumulative action of all antioxidants present in the plasma and body fluids, being an integrated parameter, rather than a simple sum of measured antioxidants (Díaz et al. 2004; Winston et al. 2004; Paital and Chainy 2010). The antioxidant activity of astaxanthin has been reported to be 100–500 times stronger than tocopherols in preventing lipid peroxidation in rat mitochondria (Kurashige et al. 1990). Winston et al. (2004) found that astaxanthin plays an important role in protecting grass shrimp *Palaemonetes pugio*

*Corresponding author. Email: acdiaz@mdp.edu.ar

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embryos from oxidant damage in the late embryogenesis when organelles have become differentiated.

In penaeids, astaxanthin is the predominant carotenoid; it comprises about 90% of the total pigments in kuruma prawn *Marsupenaeus japonicus* (Ishikawa et al. 1966) and 86–98% in the exoskeleton of black tiger prawn *Penaeus monodon* (Okada et al. 1994). Astaxanthin is present as free astaxanthin, esterified astaxanthin (astaxanthin monoester and astaxanthin diester) or bound to protein as carotenoprotein (Katayama et al. 1971). The distribution of these forms of astaxanthin also varies with species, life history stages and the organ or tissue (Okada et al. 1994; Dall 1995; Pètit et al. 1998; Pan and Chien 2000). As crustaceans are unable to synthesize carotenoids de novo, they rely upon the diet as the source of these compounds (Meyers and Latscha 1997). The total carotenoid content of juvenile *Pleoticus muelleri* fed diets supplemented with β-carotene was between 18 and 28 $\mu g\, g^{-1}$ dry weight (Díaz et al. 2007). In other penaeid species, carotenoid values between 4 and 18 $\mu g\, g^{-1}$ dry weight were determined (Gopakumar and Nair 1975; Yanar et al. 2004).

The life cycle of *P. muelleri* occurs completely in the sea without entering coastal estuarine regions, the shrimp has a large distribution area in south-west Atlantic waters from 20°S, Espíritu Santo, Brazil, to 50°S, Santa Cruz, Argentina (Díaz 2001). Larval development comprises six naupliar stages, three protozoea stages and two mysis stages (Iorio et al. 1990). The postlarval stage extends up to the moment when larva reaches 20 mm total length (Boschi 1989). Under intensive culture conditions, at 20°C, the naupliar stage lasts from 2 to 3 days, protozoea stage between 7 and 10 days and mysis stage between 5 and 8 days, reaching the first postlarva at 14–21 days (Mallo et al. 1999).

In crustaceans, the ontogeny of carotenoid metabolism remains an unsolved problem, probably due to the complexity of embryonic and larval periods. However, there is no doubt that the series of morphological, physiological and ethological changes that occurs is closely related to the biochemical evolution of pigmentation (Mantiri et al. 1995; Berticat et al. 2000). The major lipoglycoprotein in the yolk is vitellin, which is a high-density lipo-glyco-carotenoprotein (Sagi et al. 1995). Crustacean eggs accumulate carotenoids in significant amounts, suggesting possible functions as protection against radiation, as antioxidants and as a source of provitamin A (Menasveta et al. 1994; Dall 1995).

The objectives of this study were to contribute to the knowledge of carotenoid metabolism during ontogeny, and to determine the free radical scavenging properties at different larval and postlarval stages in the shrimp *P. muelleri*.

Materials and methods

Animal supply and maintenance

Specimens of *P. muelleri* were reared from hatchery-raised postlarvae (wild broodstock from Mar del Plata, Argentina) at Nagera Station, Marine Science Department, Mar del Plata National University, Argentina. Nauplii were transferred to 3.5 ton upwelling tanks and maintained at 21°C (±1°C). Larvae were reared on a diet of algae (*Chaetoceros gracilis*, *Tetraselmis chuii*) and *Artemia salina* nauplii. During the larviculture, the animals were fed commercial microencapsulated feeds (Frippak feeds) of different particle sizes: protozoea 1 (0.30 μm), protozoea 2 and 3 (30–90 μm), mysis and early postlarvae (80–150 μm) (Mallo et al. 1999).

Carotenoids analysis

Whole larvae and postlarvae were lyophilized and homogenized under 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 under argon atmosphere in darkness, and astaxanthin was separated by partitioning with dimethyl sulphoxide (DMSO)/acetone (1:3) until colourless under inert atmosphere.

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). The concentrations of carotenoids were calculated employing standard curves of β-carotene in hexane (1.88×10^{-6} M) and astaxanthin in DMSO/acetone (4.19×10^{-6} M); the absorbance were read at 466 and 472 nm, respectively, with extinction coefficients of 122,000 and 124,000 $M^{-1}\, cm^{-1}$ (Perkampus 1992).

Electronic paramagnetic resonance (EPR) measurements

The potential antioxidant activity of different larval stages: nauplius (N), protozoea (Pz), mysis (M) and postlarvae of 1, 6, 10, 26, 30 days (PL1, PL6, PL10, PL26, PL30) was investigated on the basis of the scavenging activity on the stable 1,1-diphenyl-2-picryhydrazyl radical (DPPH). About 20 mg of tissue was mixed with 1 ml of chloroform under an argon atmosphere. Aliquots of 100 μl with 100 μl of DPPH solution (1.26×10^{-4} M) at 20°C were analyzed at fixed time intervals of 5, 10, 30 and 60 min. Control or blank preparations, containing 100 μl of DPPH solution (1.26×10^{-4} M), prepared under argon, remained unaltered after a long period of time. DPPH proved to be a very stable free radical (Díaz et al. 2004); the intensity of the signal, measured in arbitrary units,

decays with time and represents the radical-tissue reaction.

To analyze the presence of radicals at the different larval and postlarval stages, tissue samples of 20 mg were mixed in 200 µl of chloroform under an argon atmosphere. Aliquots of 100 µl with 100 µl of chloroform were analyzed at fixed time intervals for 72 h.

A Bruker ER 200D spectrometer with a rectangular TE102 resonance cavity was used for recording X-band EPR spectra from different samples. The measurements were repeated at least three times to minimize random errors. The standard plotting program Origin was used to fit all sets of points whenever necessary.

Statistical analysis

One-way analyses of variance (ANOVA) were used to test significant differences in tissue carotenoid content among the different developmental stages. Data were expressed as mean ± standard error. Pearson's rank correlation coefficient was used to identify significant correlations among carotenoids content. In all cases, significance was set as $P < 0.05$ (Sokal and Rohlf 1995).

Results

Carotenoid analysis

Carotenoid concentrations found for the different stages are summarized in Figure 1. The quantitative analysis of the carotenoid content of the whole larvae and postlarvae, expressed in $\mu g\,g^{-1}$of dry weight, indicated concentration values ranging between 1.72 to 87.04 $\mu g\,g^{-1}$ of β-carotene and 2.26 to 19.86 $\mu g\,g^{-1}$ of astaxanthin. The lowest value was reported in the naupliar stage, with a progressive increase of the carotenoid level up to PL6. A noticeable decrease of pigment concentration was observed within the period between the stages PL10 to PL30. β-carotene represented the major fraction of carotenoid composition, while astaxanthin occurred in very low amounts. Nevertheless, in PL30 the astaxanthin concentration (5.88 $\mu g\,g^{-1}$) appeared to be significantly higher than the concentration of β-carotene (1.73 $\mu g\,g^{-1}$).

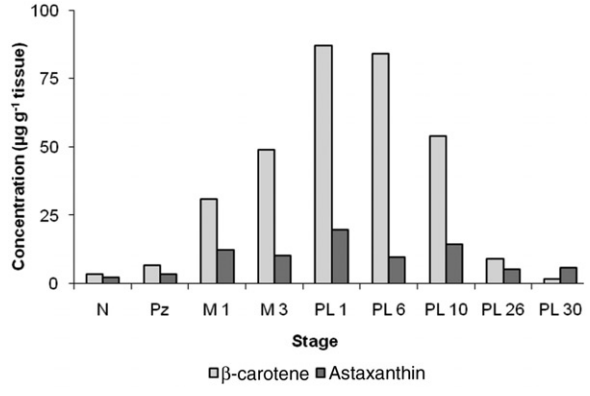

Figure 1. Variation of β-carotene and astaxanthin concentrations during larval and postlarval development in *P. muelleri*. N: nauplius, Pz: protozoea, M: mysis, PL: postlarvae.

Electronic paramagnetic resonance (EPR) measurements

Figure 2 shows the decay of the DPPH in the tissue homogenates. Significant DPPH radical scavenging capacity was detected in all tested stages; DPPH signal decayed drastically within 15 min and was effectively consumed in about 1 h. These results suggest that the tissues investigated contain natural antioxidants. There were statistical differences in the activity of different stages; postlarvae and naupliar stages showed a very high activity. On the other hand, in Pz and M stages, the DPPH radicals remained without reaction, approximately in a 50%, even after an hour.

The next step of the study was to look for a pool of free radicals in the tissue homogenate. It was recorded as typical wide and undefined signal in the concentrated homogenates (0.1 mg μl^{-1}) of larvae and postlarvae, which suggested the presence of some persistent radical types in the tissues. Figure 3 shows the recorded spectra of M, PL1 and PL30; a concentration of about 10^{-5} M was estimated. The highest concentration was measured in M stage (1×10^{-5} M). During the 72 h of this trial, the signal persisted.

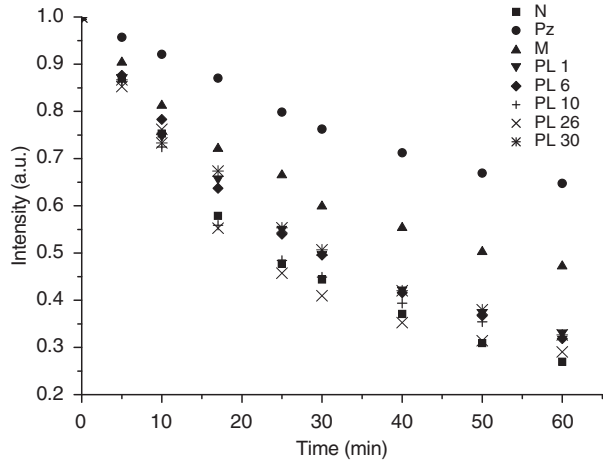

Figure 2. DPPH scavenging activity of *P. muelleri* tissue following up to 60 min. Different stages of development were tested at room temperature by mixing 100 µl of homogenate (20 mg ml^{-1}) with 100 µl of DPPH solution (126 µM). The signal intensity in arbitrary units represents the remaining DPPH. N: nauplius, Pz: protozoea, M: mysis, PL: postlarvae.

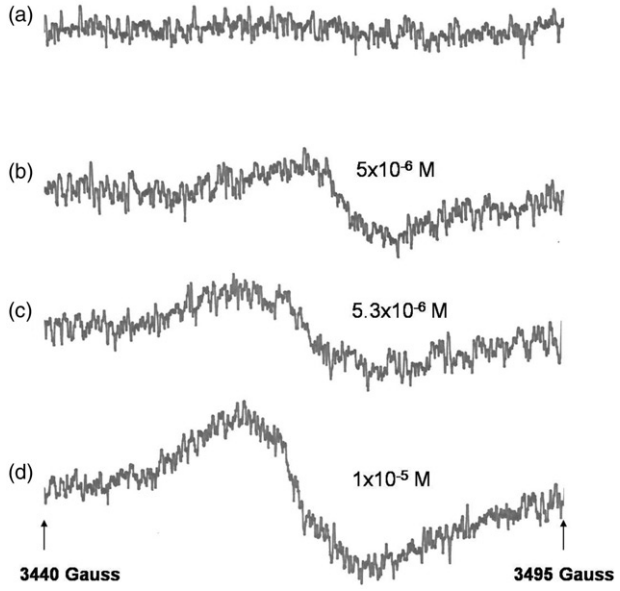

Figure 3. EPR spectra of concentrated homogenates ($0.1\,mg\,\mu l^{-1}$) of *P. muelleri* larvae and postlarvae showing the signal of pool of radicals. a: baseline (chloroform); b: PL30; c: PL1; d: M.

Discussion

Antioxidants provide some of the protective effect, but there are many other constituents that may exert additional antioxidant effects, or protect by completely different mechanisms. In term of antioxidants, particular attention has been paid to the carotenoids and plant phenolics, although evidence that they are important antioxidants in vivo is limited as yet (Halliwell and Gutteridge 2001). Amar et al. (2001) indicated that the carotenoid supplemented diets have a beneficial effect on bio-defence mechanisms in rainbow trout and found that the serum complement activity in astaxanthin-supplement fed groups were significantly higher than in control fish. In vitro studies also showed the potential of carotenoids to act as free radical scavengers (Liebler and McClure 1996); oxidizing radicals can react with carotenoids by electron transfer; however, it is uncertain how important this would be to healthy animals.

It has been reported that antioxidants such as tocopherols and propylgallate either delay or inhibit the initiation and propagation stages of oxidation by reacting with lipid-free radicals and peroxy or alkoxy radicals, respectively. Carotenoids have been reported to be the most efficient molecules for $^1O_2$ quenching. Astaxanthin as a member of carotenoid family has conjugated double bonds and phenolic hydroxyl groups, which may reduce the oxidation rate (Pu et al. 2010). Our results showed a good relationship between the carotenoid concentration and the protective capacity of the homogenate. The postlarval stages (with higher carotenoid concentration) exhibited a greater percentage of decay of the DPPH over time. It is known that when a solution of radical DPPH is added to the extract of a tissue, it is consumed with a speed that depends on the amount of protective substances. An exception was observed in the naupliar stages, with the minor total carotenoid concentration and the greatest protective activity; only 2% of DPPH remained after 60 min. Nauplius larvae are lecitotrophic; they depend upon vitellin reserves and do not assimilate exogenous pigments. Carotenoids have been suggested to have the capacity to trigger shrimp vitellogenesis, and the effect is directly related to the transcription of hormone genes directly involved in maturation of the ovary (Liñan-Cabello and Paniagua-Michel 2004). Liñan-Cabello et al. (2004) reported the positive effect of carotenoids in the ontogenic development of oocytes in female *Cherax quadricarinatus*. It has been hypothesized that reductions in larvae quality were caused by reduced carotenoids levels in the yolk of developing shrimp embryos (Regunathan and Wesley 2006). However, the present results do not support this conclusion; in the shrimp *P. muelleri* other unidentified protective substances are involved besides carotenoids.

As the larvae begin to feed themselves and incorporate carotenoids with the diet, they use either phytoplankton alone or a combination of phyto- (*C. gracilis* and *T. chuii*) and zooplankton (*Artemia salina* nauplii) as their major food. In the present study, a progressive increase of carotenoid concentration in Pz, M, PL 1, PL 6 and PL 10 was observed. Under culture conditions, *P. muelleri* from PL10–12 begins to feed artificial diet at the bottom of the tank (microencapsulated feeds), due to the behavioural changes that happen during the important transition from planktonic to benthic life style that takes place after the metamorphosis (Díaz et al. 2008). The ability to use the carotenoids present in food is limited by the genotype and physiology of the animal in question. In Penaeidae, the ability to metabolize precursor pigments appears in the postlarval stages and is linked to the development of oxidation and esterification pathways of carotenoids which produce metabolic intermediates related to the biosynthesis of astaxanthin from alimentary precursors. This could be the reason for the diminution of the β-carotene concentration detected in *P. muelleri* after PL10 stage (Figure 1). This fact has been studied by experiments using labelled pigments (Katayama et al. 1972). Also, in juveniles *Litopenaeus vannamei* co-cultured with green seaweed *Ulva clathrata* in outdoors tanks, it has been shown that carotenoids present in seaweeds, luteins principally, were efficiently assimilated, metabolized into astaxanthin and deposited by the shrimp (Cruz-Suárez et al. 2010). Pètit et al. (1991) showed that while astaxanthin is deposited as the free form in

M. japonicus larvae, it occurs increasingly as esterified forms in late postlarval stages. Lotocka et al. (2004) has studied developmental groups of copepod *Pseudocalanus acuspes* and *Acartia* spp. and demonstrated that astaxanthin decreased with ontogeny of the copepods, while astaxanthin esters increased. These studies on using esterified forms as metabolic intermediates are not complete and can only be confirmed by more sophisticated research.

In Pz and M stages it was observed the lowest scavenging capacity of DPPH. According to Díaz et al. (2004), the shrimp midgut gland is the main site of antioxidant activity. Previous histological studies in *P. muelleri* showed that the organogenesis has not been completed at early larval stages (Pz and M); the main digestive enzyme secretion sites are the anterior and lateral midgut caeca (Díaz et al. 2008). This fact would explain the low scavenging activity in these stages.

The existence of pool of radicals in vivo will depend on the balance between diverse processes: those that lead towards the formation of protective substances, those that produce reactive radicals and those that generate persistent radicals. The protective substances neutralize the very reactive radicals transforming them into metabolizable species or forming persistent radicals, as was observed in the present study (Figure 3). The protective capacity of the endogenous antioxidant system of *P. muelleri* to neutralize the DPPH radical can be quantified and provides with a very useful index to measure the relative susceptibility of biological tissues to the damage caused by the free radicals. Therefore, it would be appropriate to investigate the composition of the extracts and identify the compounds that would be responsible for the antioxidant activity.

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