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Carotenoids and their derivatives in organs of the maturing female crayfish *Cherax quadricarinatus*

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Carotenoids were separated by high-performance liquid chromatography from organ homogenates of pre-vitellogenic (oocyte diameter ~300μm) and late-vitellogenic (oocyte diameter >1mm) *Cherax quadricarinatus* females. Carotenoids were present predominantly in the cuticle and in the late-vitellogenic ovary. Smaller amounts were present in the hepatopancreas, and almost no carotenoids were found in the muscular tissue. The cuticle contained mostly esterified astaxanthins. The ovaries contained mostly non-esterified astaxanthin. Pre-vitellogenic ovaries were also relatively rich in lutein, whereas late-vitellogenic ovaries were relatively rich in β-carotene. The hepatopancreas contained mostly β-carotene. Possible roles for the different carotenoids in the cuticle and ovary of the crayfish are discussed.

Key words: Carotenoids; *Cherax quadricarinatus; Decapoda;* Crustacea; Cuticle; Ovary; Gonad maturation.

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Introduction

Cherax quadricarinatus is among the largest of the fresh water Australian crayfish. It has thus attracted considerable interest as a potential aquaculture species, and efforts have been made to culture it on an artificial diet. An understanding of the dietary needs of *C. quadricarinatus* is thus essential if this species is to be grown and bred in captivity. Crustaceans cannot synthesize carotenoids, *de novo* but can alter dietary carotenoids by oxidation and deposit them in their tissues. Carotenoids are distributed in several parts of the crustacean body (see review by Ghidalia, 1985).

Carotenoids are among the most widely distributed class of pigments found in nature, being present in microorganisms, plants and animals. Carotenoids are known to serve as colorants and anti-oxidant agents (Yamada *et al.*, 1990; Miki, 1991) and are thought to play a role in crustacean reproduction (Gilchrist and Lee, 1972). Keto carotenoids act as skin and flesh pigments in several classes of animal (Grangaud *et al.*, 1963; Choubert, 1981), including the decapod crustaceans (Gilchrist and Lee, 1972; Chien and Jeng, 1992). Astaxanthin has been found to be the predominant pigment in the cuticle of the shrimp (Schiedt *et al.*, 1993).

Oogenesis in crustaceans is characterized by rapid deposition of yolk in the oocyte, which results in a fast increase in oocyte diameter. The yolk contains proteins, lipids and carbohydrates (Adiyodi, 1985). The major lipoglycoprotein in the yolk is vitellin, which is accumulated in the oocyte cytoplasm and is later used as a source of nutrition for the developing embryo. The crustacean vitellin is a high-density lipoprotein frequently associated with carotenoids (Wallace *et al.*, 1967). Recently, it has been suggested that the purified vitellin of a number of crustaceans is in fact

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a lipo-glyco-carotenoprotein (Chang *et al.*, 1993a,b).

To evaluate the physiological, somatic and reproductive importance of carotenoids in crayfish, we studied carotenoid content and distribution in different tissues (hepatopancreas, ovary, cuticle and tail muscle) of previtellogenic and late-vitellogenic *C. quadricarinatus* females.

Materials and Methods

Animals

C. quadricarinatus juveniles were bred at the laboratories of the Agricultural Research Organization at Beit Dagan, Israel, and reared at Ben-Gurion University. Young females were separated from the population and reared in aquaria at 27 ± 2°C until they reached the age of 5–8 months. Water quality was controlled by recirculating the water through a gravel biofilter. The crayfish were fed daily with frozen ground fish (Nile Perch) and vegetables (carrot and corn). Total body weight of each animal was recorded. The ovaries were dissected out, and the wet weight of each dissected organ was recorded and used for the calculation of the gonadosomatic index (GSI = gonad wt./body wt. × 100). Oocyte diameter was measured using fresh oocytes (a sample of ≥15 oocytes per ovary) under a light microscope.

Organ samples (hepatopancreas, muscle, cuticle and ovary) were taken from previtellogenic and late-vitellogenic females, immediately immersed in pure acetone and stored on ice in the dark.

Extraction of carotenoids

Samples were homogenized in acetone by an Ultra-turax (Janke & Kunkel, GMBH) T25 homogenizer and diluted with water to a final concentration of 80% acetone. The carotenoids were extracted into hexane. If the carotenoid concentration was too low for analysis, the hexane phase was dried completely under nitrogen, and the residue was redissolved in hexane. The samples were filtered through a 0.45-μm filter before high-performance liquid chromatography (HPLC) analysis.

HPLC analysis

HPLC analysis was performed using a Varian (Barspec, Israel) 5000 LC system equipped with a Barspec Chromoscope fast scanner detector and a 250 × 4 mm (id) Lichrospher 100, RP-18, 5 μm column (Merck). A stepwise elution program with two solvents, 75:25 (v/v) methanol:water (solvent A) and ethyl acetate (solvent B) was used. The development time of 40 min was made up as follows: a linear gradient from 30% to 55% B (made up with A) for 10 min, 55% B for 5 min, a linear gradient of 55–75% B for 8 min, 75% B for 7 min, a linear gradient of 75–30% B for 4 min and 30% B for 6 min. Injection volume was 20 μl, and flow rate was 0.6 ml/min at 35°C. The absorbance data were collected and integrated by the Barspec Data System. Pigments were identified at 470 nm by their retention times (RT) and by absorbance spectra in comparison with the following known markers; β-carotene and lutein (xanthophyll) (Sigma, St Louis, MO, U.S.A.) and astaxanthin (Hoffmann-La Roche, Basel, Switzerland). The absorption spectra was carried out concurrently during each HPLC run. Quantitative determination was performed by peak area integration, calibration being performed against the absorbance of known concentrations of the above pure standards.

Results

The diameter of the oocytes in the ovaries of late-vitellogenic *C. quadricarinatus* females was over three times that of the previtellogenic females (1022 and 258 μm, respectively). The gonado-somatic index for females with ripe ovaries was four times higher than that for crayfish with pre-vitellogenic ovaries. The amount of carotenoids accumulated per organ in the ripening ovary was greater, by two orders of magnitude, than that in the previtellogenic ovary (Table 1).

Representatative HPLC chromatograms of the carotenoid profile from the ovary, cuticle and hepatopancreas of late-vitellogenic female are given in Fig. 1 (A, B and C, respectively). In the ovary the major peak of RT 31.6 min represents β-carotene. Peaks of 11–14.5 min represent free astaxanthin derivatives, and the small peak at 14.9 min represents lutein (Fig. 1A). In the cuticle all of the peaks are astaxanthin derivatives; RT of 11–14.5 min represents free astaxanthins and the remainder of the peaks represent monoester (RT 23–30 min) and diester (RT 30.4–37 min) astaxanthins (Fig. 1B). The predominant peak in the hepatopancreas is that of β-carotene. The small peak at RT 11.5 min represents free astaxanthin (Fig. 1C).

The content and distribution of carotenoids in the different tissues are presented in Table 2. The cuticular carotenoids were solely astaxanthin, some of which was present as free astaxanthin but most of which was esterified with fatty acids. No significant differences in cuticular carotenoid distribution were found

Table 1. Reproductive properties and ovarian carotenoid content of pre- and late-vitellogenic *C. quadricarinatus* females

	Pre-vitellogenic females (n = 5)	Late-vitellogenic females (n = 5)
Gonado-somatic index	0.37 ± 0.12	2.15 ± 0.14
Oocyte diameter (μm)	258 ± 7.6	1022 ± 37.4
Total ovarian carotenoids (μg/organ)	0.20	36.07

Data are presented as mean values ± SEM. The differences between pre- and late-vitellogenic ovary were found to be statistically significant (t-test; $P < 0.001$). Gonado somatic index = gonad wt./body wt. × 100. Oocyte diameter was calculated using a random sample of 15 oocytes per ovary. The carotenoid content was calculated from a representative HPLC chromatogram for each female type.

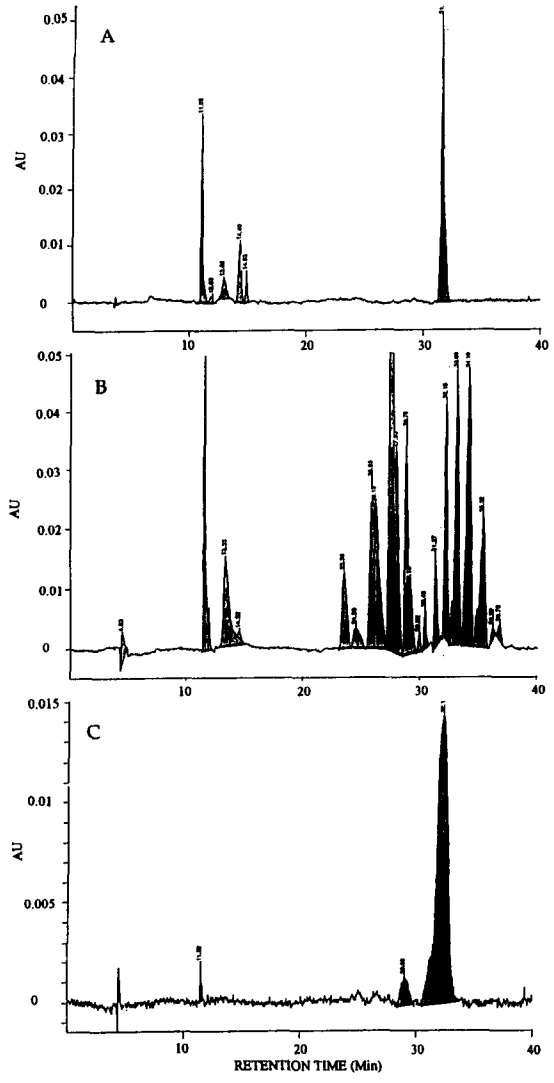

Fig. 1. HPLC chromatograms (at 470 nm) of carotenoids extracted from ovary (A), cuticle (B) and hepatopancreas (C). The extracts were taken from a late-vitellogenic female. The peak at RT 11 min represents free astaxanthin. The ovarian (A) and hepatopancreatic (C) peaks at 31.6 min represent β-carotene, and the abundant cuticular (B) peaks between RT 23 min and RT 37 min represent esterified astaxanthin derivatives.

between the pre- and late-vitellogenic females. The hepatopancreas contained mainly β-carotene and some free astaxanthin; in the pre-vitellogenic females some unidentified carotenoids (RT 21 and 24 min) were also found. In the ovary more than 60% of the carotenoid content was in the form of free astaxanthin, the remainder being made up of β-carotene and other xanthophylls (predominantly lutein). No esterified astaxanthin was found. The concentration of carotenoids in the late-vitellogenic ovary was ~5-fold that in the pre-vitellogenic ovary. The late-vitellogenic ovary was relatively rich in β-carotene, whereas the pre-vitellogenic ovary was relatively rich in lutein.

Discussion

The two main target tissues for carotenoid accumulation in *C. quadricarinatus* females were the ovary and the cuticle. However, there were significant differences in the carotenoid composition of these two organs, which have different physiological functions. As is the case for crabs (Gilchrist and Lee, 1972), the cuticle contained mostly astaxanthins, whereas the vitellogenic ovary also contained β-carotene in significant amounts. Furthermore, astaxanthin in the cuticle was mostly in the esterified form, whereas there was no esterified astaxanthin in the ovary. These differences may be a reflection of different roles for specific carotenoids in the different organs.

The function of carotenoids in living organisms is not fully understood. However, all carotenoids are known to act as powerful antioxidants in biological membranes (Miki, 1991). In addition. β-carotene is the most efficient provitamin A (Bouernfeind, 1972; Olsen, 1989), and astaxanthin serves as a pigment component in tissues (Grangaud *et al.*, 1963; Choubert, 1981).

We found that the cuticle contained mainly the monoesters and diesters of astaxanthins.

Table 2. Carotenoid content and distribution in different tissues of *C. quadricarinatus* females

Organs	Ovarian stage	Astaxanthin(%) Free	Monoester	Diester	Astaxanthin (total %)	β-carotene (%)	Lutein (%)	RT 21,24 (%)	Carotenoids (total μg/g)
Hepatopancreas	PV	4.6	0	0	4.6	81.9	ND	13.5	8.42
	LV	1.5	0	0	1.5	98.5	ND	ND	1.33
Muscle	PV	14.6	45.5	30.1	90.2	9.8	ND	ND	0.62
	LV	100.0	0	0	100.0	ND	ND	ND	0.04
Cuticle	PV	19.5	50.0	30.5	100.0	ND	ND	ND	43.24
	LV	19.6	49.4	31.0	100.0	ND	ND	ND	51.22
Ovary	PV	63.7	0	0	63.7	9.0	27.3	ND	7.17
	LV	66.8	0	0	66.8	23.8	9.4	ND	34.43

Carotenoids were extracted with acetone from homogenized tissues of representative pre-vitellogenic (PV) and late-vitellogenic (LV) females and analyzed using HPLC system. ND, not detectable.

Such compounds have been found in green alga grown under high light (Donkin, 1976; Czygan, 1982; Grung *et al.*, 1992; Rise *et al.*, 1994). In algae, centrally located astaxanthins disperse toward the periphery of the cell under light induction and move back toward the center after illumination is discontinued (Yong and Lee, 1991). It has thus been assumed that the esterified astaxanthins have a photoprotective role by a "passive" mechanism, that is, they act as filters or sunscreens (Bidigare *et al.*, 1993). It is thus possible that the esterified astaxanthin in the crab (Gilchrist and Lee, 1972), the shrimp (Shiedt *et al.*, 1993) and the crayfish cuticle may play a role in the prevention of over-exposure to radiation via light-dependent dispersion of the pigments in the chromatophores (see review by Rao, 1985). The non-polar nature of the esterified carotenoids enables their accumulation in fat globules in the cuticle (see review by Ghidalia, 1985). Ghidalia (1985) also proposed two possible protective roles of carotenoids, in addition to protection against high radiation, they also confer environmental protection in the form of coloration that matches the habitat, thus allowing the crayfish to blend with its surroundings.

The greater accumulation of total carotenoids in late-vitellogenic crayfish ovaries (vs pre-vitellogenic ovaries) was correlated with the development of the oocyte and the accumulation of vitellin in the ovaries (see review by Quackenbush, 1991). Ovarian carotenoids have been suggested to be associated with proteins in a form of lipo-glyco-carotenoprotein complex (Gilchrist and Lee, 1972; Chang *et al.*, 1993a,b). Thus, the free form of astaxanthin found in the crayfish vitellogenic ovary could be a component of the carotenoprotein lipovitellin molecule (see review by Ghidalia, 1985).

Three possible roles may be suggested for carotenoids in the ovary. (1) Because the egg shell in the crayfish is transparent, the accumulation of carotenoids in the oocyte may provide protection against over-radiation. (2) Carotenoids may have a metabolic role in the ovary and eggs (e.g., as antioxidants or provitamin A). (3) In crayfish such as *C. quadricarinatus*, in which no feeding larval stages exist and young crayfish are hatched from the eggs in the form of juveniles, carotenoids are accumulated in the oocyte to suffice the embryonic need for pigmentation in the construction of the cuticle. Thus, it may be suggested that vitellin serves as carrier of the hydrophobic astaxanthin, whereas astaxanthin protects vitellin against oxidation.

Astaxanthin may be absorbed by crustaceans from their diet, and some crustaceans are also able to convert β-carotene to astaxanthin (Yamada *et al.*, 1990). The biosynthetic pathway of astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) has not yet been fully elucidated. However, it must undergo hydroxylation in positions 3 and 3′ of the β ionone ring, followed by oxidation to form the keto groups in positions 4 and 4′. It is thus possible that astaxanthin is synthesized via canthaxanthin (β,β-carotene-4,4′-dione) or via zeaxanthin (β,β-carotene-3,3′-diol) (Tanaka *et al.*, 1976; Grung *et al.*, 1992). It is also possible that lutein (hydroxylated α-carotene) serves as a precursor for astaxanthin (Katayama *et al.*, 1970). The conversion site of ovarian astaxanthin has yet to be discovered. Dietary β-carotene is converted to astaxanthin in the hepatopancreas, from which it is immediately transported to the ovary. This may explain the small concentration of astaxanthin in hepatopancreas, especially in late-vitellogenic females. On the other hand, lutein may serve as a precursor for astaxanthin biosynthesis. Thus, the presence of lutein in the ovary suggests that the conversion to astaxanthin may take place in the ovary.

This is the first study of the distribution of carotenoids in the somatic and reproductive tissues of the adult crayfish *C. quadricarinatus*. Future studies of carotenoid metabolic pathways in the crayfish body may prove in-

strumental in the understanding of the dietary needs of *C. quadricarinatus* for growth and reproduction in captivity.

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