# Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia*

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**Abstract** In order to study the biological fate of all-*trans*- and *cis*-canthaxanthin in the brine shrimp *Artemia*, a quantitative method was developed for the determination of both carotenoids and their metabolic precursors in encysted embryos (cysts), nauplii, whole animals, organs, and subcellular fractions. This method is based on nonaqueous reversed-phase chromatography, two new exhaustive extraction procedures, and the determination of proteins in the extracted residue. Hydration of *Artemia* cysts caused a reversible conversion of part of the all-*trans*- to *cis*-canthaxanthin. During further pre-emergence embryonic development, there was little change in the levels of both isomers. After hatching of cysts, *cis*-canthaxanthin was progressively isomerized to the all-*trans* form, while the total (all-*trans* + *cis*) canthaxanthin to protein ratio tended to remain constant. *Cis*-canthaxanthin rapidly became undetectable in animals fed on algae and reappeared in females at an advanced stage of the reproductive cycle. All-*trans*-canthaxanthin remained present during the whole *Artemia* life cycle in addition to its metabolic precursors echinenone and β-carotene. The carotenoid distribution in organs and subcellular fractions indicated high affinity of *cis*-canthaxanthin for the female reproductive system, oocytes in general, and yolk in particular. A role for *cis*-canthaxanthin is suggested at an early developmental stage, i.e., in cysts, before hatching. —**Nelis, H. J. C. F., P. Lavens, M. M. Z. Van Steenberge, P. Sorgeloos, G. R. Criel, and A. P. De Leenheer.** Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia*. *J. Lipid Res.* 1988. **29:** 491–499.

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Our previous investigations on the carotenoid composition of the brine shrimp *Artemia* led to the discovery of hitherto unrecognized *cis*-carotenoids (*cis*-canthaxanthins) in this organism (1). Preliminary data suggesting an unexpected selectivity in the appearance/disappearance and distribution of these unusual pigments (1) warranted the development of a method for their quantitative determination. Current assays for canthaxanthin in *Artemia* all rely on spectrophotometry in conjunction with open-tubular column chromatography or thin-layer chromatography, but fail to distinguish between all-*trans*- and *cis*-canthaxanthin (2–5). Most studies in this area have been concerned with the biochemical pathways of carotenoid metabolism in *Artemia* (2–4). No attempts have been made so far to relate qualitative and quantitative changes in the carotenoid pattern with specific developmental stages.

In this report we describe a method based on nonaqueous reversed-phase HPLC (1, 6, 7) and two new exhaustive extraction procedures for the quantitation of all-*trans*- and *cis*-canthaxanthin as well as their metabolic precursors β-carotene and echinenone in small samples (mg amounts) of *Artemia*. We have applied this method to encysted embryos (cysts), nauplii, whole animals, organs, and subcellular fractions to study the changes in both isomeric canthaxanthin forms during *Artemia* development.

## MATERIALS AND METHODS

### Chemicals

All-*trans*-canthaxanthin, echinenone, β-*apo*-8'-carotenal and β-*apo*-8'-carotenoic acid ethyl ester were gifts from Hoffmann-La Roche (Basle, Switzerland). β-Carotene was purchased from Fluka (Buchs, Switzerland). *Cis*-canthaxanthin (collective term for a mixture of one predominating and two minor *cis* isomers, substantially free from all-*trans*-canthaxanthin) was prepared as

Abbreviations: HPLC, high performance liquid chromatography; RSD, relative standard deviation; PMS, post-mitochondrial supernatant.

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described previously (1). All chemicals were reagent grade except acetonitrile, methanol, and dichloromethane which were "chemically pure." The latter two were redistilled before use in a spinning band apparatus. Bond Elut $C_{18}$ mini-columns (500 mg, 6 ml) were obtained from Analytichem.

### Biological samples

*Artemia* cysts came from the collection at the Artemia Reference Center (State University of Ghent, Gent, Belgium). Batch numbers refer to this collection. Dehydrated cysts were obtained by drying 10-mg samples in an oven at 40°C for 24 hr, followed by cooling in a desiccator over $CaCl_2$. Hydration was achieved by soaking dehydrated cysts in water for 5 hr at room temperature (20–22°C), using small plastic filter cups (Beckman). Decapsulation was carried out according to the method of Bruggeman, Sorgeloos, and Vanhaecke (8). This removal of the inert cyst shell allows one to focus exclusively on changes in the embryo itself. Nauplii were obtained by incubating cysts under standard conditions in sea water (35 g of NaCl/l). For analysis, hydrated cysts and nauplii were collected on a 120-$\mu$m sieve (9). All developmental experiments were carried out with *Artemia* from the San Francisco Bay (SFB) strain (California). Hatching for this strain was initially observed after 15 hr, but sufficient nauplii could only be collected after 16–17 hr (20% hatching). The animals were transferred to 2-liter aquaria and fed twice a day (morning and late afternoon) on living *Dunaliella tertiolecta* algae under standard culture conditions (10), until they reached the mature stage. Samples were taken on different days before the afternoon feeding and immediately frozen. Organs (shell gland, ovaries, gut) and other body sections (oocytes, hemolymph, and the remaining carcass) were removed from female adult animals, both vitellogenic and non-vitellogenic. The isolated tissues of 10–20 animals were pooled and analyzed immediately. Crude subcellular fractions were prepared from hydrated cysts as described previously (1), except that for lipovitellin extraction from yolk platelets an approximately 10 times higher ratio of extraction buffer (2 M NaCl in 0.05 M Tris, pH 9) to protein was used.

### Chromatography

The chromatographic system optimized for maximal *cis/trans*-canthaxanthin resolution has been described previously (7).

### Extraction of carotenoids from *Artemia*

Extraction of carotenoids from *Artemia* was performed with methanol, either in the presence of formic acid (method 1) or after pretreatment of the sample with a detergent (method 2). Concomitant thorough mechanical homogenization in a Potter-Elvehjem apparatus was necessary. All manipulations were carried out in subdued light, in dark brown glassware (except for the Potter tube), and at reduced temperature (ice bath).

*Method 1.* *Artemia* cysts (2–10 mg dry weight) were homogenized in 6 ml of methanol–formic acid 94:6. After neutralization of the formic acid with 1.5 ml of aqueous saturated potassium carbonate, and the addition of an internal standard ($\beta$-*apo*-8'-carotenal or $\beta$-*apo*-8'-carotenoic acid ethyl ester) the mixture was centrifuged for 2 min. A 50–100-$\mu$l aliquot of the supernatant was injected on the liquid chromatographic column. *Artemia* whole animals (a few mg of nauplii, pre-adults, adults) were first homogenized in 1 ml of methanol. After centrifugation and removal of the supernatant, the residue was re-extracted with 1.5 ml of methanol–formic acid 95:5. To the combined extracts 0.2–0.3 ml of saturated potassium carbonate was added, the mixture was centrifuged, and the supernatant was removed. Finally, the precipitate was washed with 1 ml of methanol followed by centrifugation. All supernatants were combined, the internal standard was added, and a 100-$\mu$l aliquot was injected. Isolated organs and body sections were homogenized as for whole animals. For very small samples, e.g., hemolymph, ovaries, and shell glands, the extraction was miniaturized by reducing all volumes (0.2–0.3 ml of methanol, 0.5 ml of methanol–formic acid and 0.1 ml of potassium carbonate, respectively).

*Method 2.* *Artemia* cysts (2–10 mg) were pretreated in a Potter-Elvehjem apparatus with 150 $\mu$l of 5% sodium deoxycholate and extracted with 2 ml of methanol. After centrifugation and removal of the supernatant, this step was repeated. The internal standard was added to the combined supernatants and a 100-$\mu$l aliquot was injected. In some instances, particularly when a hazy solution emerged, the addition of 1.5 ml of acetonitrile before injection was found to be helpful. For the extraction of small quantities of whole animals, including nauplii, all extraction volumes were reduced (100 $\mu$l of 5% sodium deoxycholate, 1.5 ml of methanol, and 1 ml of acetonitrile, respectively). Solid subcellular fractions, e.g., yolk platelets, can be analyzed in the same way. However, aqueous samples, e.g., PMS (post-mitochondrial supernatant), lipovitellin, total homogenates, or suspended yolk platelets, required a clean-up procedure to avoid deterioration of the column.

To this end, a 200–500-$\mu$l aliquot of suspension was mixed with 150 $\mu$l of 5% sodium deoxycholate and 3 ml of methanol. After centrifugation and isolation of the supernatant, the residue was once more extracted in the same way. Water (2 ml) was added to the combined supernatants and the mixture was applied on top of a Bond Elut $C_{18}$ mini-column, preconditioned successively with methanol and 70% methanol. The sample was forced through by applying gentle pressure, the column was

washed with 70% methanol and eluted with 5 ml of the chromatographic solvent (methanol–acetonitrile–dichloromethane 5:4:1, containing 0.15% of triethylamine). The internal standard was added and a 100-μl aliquot was injected.

### Protein determination

Proteins were determined in the solid residue remaining after extraction of the carotenoids. Alternatively, aqueous samples, i.e., suspensions of subcellular fractions, were directly analyzed for protein content after appropriate solubilization. Method 1 for extraction was combined with a protein assay based on alkaline hydrolysis (11) and determination of amino acids (12). In Method 2, the proteins were determined according to a modified Lowry procedure (13).

*Method 1.* The residue in the Potter tube containing the proteins and solid potassium carbonate was first washed with a mixture of 1 ml of 0.4 M HCl and 1 ml of 5% sulfosalicylic acid in 0.12 M HCl to remove any residual free amino acids. After 20 min standing in ice, the mixture was centrifuged, the supernatant was discarded, and the precipitate was transferred to a 100-ml TPX (polymer resistant to base and 160°C) volumetric flask with 20 ml of hot 13.5 M sodium hydroxide. Alternatively, barium hydroxide can be used (14). Hydrolysis was conducted in an autoclave at 120°C for 45 min, followed by 20 min of steaming (14). After cooling, 34 ml of glacial acetic acid was added and the volume was brought to 100 ml with distilled water. For the color reaction, 1 ml of sample was mixed in a polypropylene tube with 0.5 ml of 4 M lithium acetate buffer and 1.5 ml of ninhydrin reagent (12).

*Method 2.* The solid residue remaining after extraction or a 0.2–0.5-ml aliquot of an aqueous suspension of the subcellular fraction was solubilized with 10 ml of 3.3% SDS (sodium dodecyl sulfate) in 0.27 M NaOH. The solution was quantitatively transferred to a 50-ml volumetric flask and brought to volume with distilled water. A 0.5–1.0-ml aliquot was used for the protein determination according to the method of Peterson (13), except for the deoxycholate/trichloroacetic acid precipitation step, which was omitted. The contents of SDS and NaOH in reagent A were reduced so as to compensate for the amounts already present in the solubilization mixture.

### Quantitation

Quantitation of all-*trans*- and *cis*-canthaxanthin was based on the determination of peak height ratios (compound of interest vs. internal standard), and comparison with a calibration curve. To construct this curve, known amounts of authentic standard compounds together with the internal standard were analyzed as the unknowns and the resulting peak height ratios were plotted versus concentration. The exact concentration of standard stock solutions was determined spectrophotometrically with calculation based on the molar absorption coefficients (125,000 (477 nm) and 80,000 (467 nm) $M^{-1} \cdot cm^{-1}$ for all-*trans*- and *cis*-canthaxanthin, respectively, in acetonitrile–methanol–dichloromethane 5:3:2). Results (mean ± SD) were expressed as μg of carotenoid per mg of protein.

### Statistics

Student's *t*-test was used to determine the significance of difference between groups of presumably normal data. Correlation analysis (only when $n > 10$) was carried out using the nonparametric Spearman test for rank correlation ($r_s$, rank correlation coefficient).

## RESULTS

### Method evaluation

*Chromatography.* Representative carotenoid profiles of *Artemia* cysts (A) and starved nauplii (B) are shown in **Fig. 1.** At these stages, very little echinenone was present and β-carotene was absent. Feeding activity resulted in the appearance of lutein, echinenone, and β-carotene (Fig. 1C).

*Extraction.* Methanol and other polar organic solvents failed to extract canthaxanthins quantitatively from *Artemia* cysts. This became evident when decapsulated cysts, in which the orange pigment is clearly exposed, were repeated-

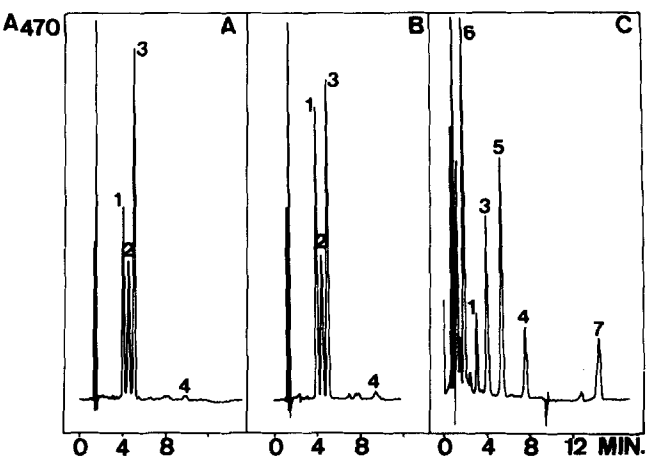


**Fig. 1.** Carotenoid patterns in *Artemia*. A, chromatogram of an extract of decapsulated cysts (SFB 522); B, chromatogram of an extract of 11-hr-old starved nauplii hatched from these cysts; C, chromatogram of an extract of growing animals (pre-adults) fed on *Dunaliella tertiolecta* algae. The column (15 × 0.46 cm) contained 5 μm Zorbax ODS. Eluent was methanol–acetonitrile–dichloromethane 5:4:1 (v/v) containing 0.15% of triethylamine; flow rate, 1.0 ml/min; detection, 470 nm. Peak 1, all-*trans*-canthaxanthin; peak 2, *cis*-canthaxanthin; peak 3, β-*apo*-8'-carotenal (internal standard); peak 4, echinenone; peak 5, β-*apo*-8'-carotenoic acid ethyl ester (internal standard); peak 6, lutein; peak 7, β-carotene.

ly treated with these solvents. We found that the addition of small amounts of formic acid (5–6%) to methanol led to an immediate and complete release of the unextracted pigment and total bleaching of the residue. However, the presence of the acid in the injected extracts adversely affected column efficiency and resolution. In addition, most carotenoids, e.g., the internal standards, are unstable in acid. Both drawbacks could be overcome by neutralizing the acid with potassium carbonate. However, despite its effectiveness and reproducibility, the formic acid approach occasionally produced anomalous results. This prompted us to develop a milder alternative extraction approach. We found that pretreatment of cysts with small volumes of a 5% aqueous detergent solution (sodium deoxycholate in particular) prior to extraction with methanol was even more effective than formic acid. The relative recovery of *cis*-canthaxanthin from cysts using different extraction strategies was 25% (no additive), 86% (formic acid method), 54% (Tween 80 pretreatment), 94% (SDS pretreatment) and 100% (sodium deoxycholate). Corresponding values for all-*trans*-canthaxanthin were 30, 76, 51, 82, and 100%, respectively. Stability of the internal standards in the sodium deoxycholate–methanol medium was excellent. Re-extraction with methanol–formic acid of the residue after detergent–methanol treatment did not yield any supplementary canthaxanthin.

As a rule, whole animals, organs and tissues and subcellular fractions were easier to extract than cysts. In method 1, pre-extraction with acid-free methanol recovered most of the canthaxanthin as well as the (acid-labile) echinenone and β-carotene. A second extraction with methanol–formic acid was still performed to recover any residual *cis*-canthaxanthin, which in general appeared to be more tightly bound than the all-*trans* form.

*Quantitation.* Upon repetitive determination of all-*trans*-canthaxanthin in nauplii, method 1 yielded RSDs ranging from 5.6% ($\bar{x}$ = 1.53 ± 0.09 μg/mg, n = 12) to 13.2% ($\bar{x}$ = 2.85 ± 0.38 μg/mg, n = 9). Corresponding values for *cis*-canthaxanthin were 6.3% ($\bar{x}$ = 0.58 ± 0.04 μg/mg, n = 10) and 13.0% ($\bar{x}$ = 1.13 ± 0.15 μg/mg, n = 10), respectively. Precision data obtained with method 2 (different samples) were as follows: all-*trans*-canthaxanthin, $\bar{x}$ = 1.65 ± 0.04 μg/mg (RSD 2.4%, n = 10) and 1.25 ± 0.045 μg/mg (RSD 3.6%, n = 10); *cis*-canthaxanthin, $\bar{x}$ = 0.69 ± 0.02 μg/mg (RSD 3.0%, n = 10) and 0.97 ± 0.03 μg/mg (RSD 3.3%, n = 10). The precision of the analysis of subcellular fractions using the solid phase clean-up approach can be assessed from **Table 1.**

The accuracy is more difficult to determine because the method deals with heterogenous samples and no reference method is available. In general, however, the exhaustive character of the extraction approaches (no supplementary pigment recovery from the colorless residue) as well as the overall simplicity of the sample pretreatment under mild conditions are likely to guarantee optimal recovery of pigments, particularly of the labile *cis*-canthaxanthin (1).

## Application

*Pre-emergence embryonic development in cysts.* The all-*trans*- and *cis*-canthaxanthin levels of fully dehydrated and hydrated cysts were significantly different. Hydration caused an increase in *cis*-canthaxanthin with a concomitant decrease in all-*trans*-canthaxanthin. This phenomenon consistently occurred in cysts from 18 different geographi-

TABLE 1. Quantitative analysis of subcellular fractions prepared from *Artemia* cysts (SFB 288/2606 and GSL Z-627)

| | Canthaxanthin Concentration (μg/mg of protein)[a] | | | |
|---|---|---|---|---|
| Subcellular Fraction | All-*Trans*-Canthaxanthin | *Cis*-Canthaxanthin | Total Canthaxanthin | *Cis*/*Trans* Ratio |
| A. Analysis of fractions without Bond Elut clean-up procedure[b] (SFB 288/2606) | | | | |
| Total cysts | 0.35 | 0.53 | 0.88 | 1.51 |
| Total homogenate | 0.33 | 0.46 | 0.79 | 1.39 |
| PMS | 0.47 | 0.32 | 0.79 | 0.68 |
| Yolk platelets | 0.24 | 0.86 | 1.10 | 3.58 |
| Insoluble fraction | 0.27 | 0.59 | 0.86 | 2.19 |
| Lipovitellin | 0.20 | 0.83 | 1.03 | 4.15 |
| B. Analysis with solid phase extraction clean-up procedure[c] (GSL Z-627) | | | | |
| Total homogenate[d] | 0.64 (0.03) | 1.07 (0.095) | 1.71 (0.12) | 1.67 |
| PMS[e] | 0.31 (-) | 0.17 (-) | 0.48 (-) | 0.55 |
| Yolk platelets[e] | 0.78 (0.028) | 1.82 (0.035) | 2.60 (0.007) | 2.33 |

[a] Procedure 2 for protein determination.
[b] Solid fractions were analyzed directly; aqueous suspensions were subjected to extraction with diethyl ether after treatment with deoxycholate–methanol.
[c] All fractions were analyzed as suspensions. Aliquots were taken for canthaxanthin and protein determination.
[d] (SD), n = 3.
[e] (SD), n = 2.

cal strains studied, although to different extents (15). The all-*trans*→*cis* conversion could be monitored as a function of hydration time; in Reference *Artemia* cysts, the *cis/trans* ratios increased from 1.02 (dehydration) to 1.28 (30 min of hydration), and reached a plateau value of 1.50 after 3 hr of hydration. The isomerization was reversed upon subsequent dehydration of the hydrated cysts, a phenomenon that could be reproduced several times (**Fig. 2**).

The evolution of the all-*trans*- and *cis*-canthaxanthin levels in (decapsulated) cysts during the whole period of pre-emergence development is shown in **Fig. 3.** In this particular experiment, the effect of initial hydration was not so clear, which is probably explained by the less vigorous dehydration procedure used (storage in brine rather than total desiccation at 40°C). Yet there was a slight increase in *cis/trans* ratio (from 0.99 to 1.07) after 2 hr of incubation. Also there may have been a partial *cis*→*trans* conversion towards the end of pre-emergence development, i.e., beyond 10 hr, a point approximately coinciding with the beginning of differentiation, when the *cis/trans* ratio dropped from 1.03 to 0.88. Overall, however, the concentrations of all-*trans*-canthaxanthin (1.14 ± 0.07 μg/mg of protein, n = 13) and *cis*-canthaxanthin (1.12 ± 0.06 μg/mg of protein, n = 13) remained practically constant during the whole period of pre-emergence development.

*Early naupliar development.* After hatching of cysts, there was a progressive decrease in *cis*-canthaxanthin with a concomitant increase in all-*trans*-canthaxanthin during the first day of development (Fig. 3). At the same time, the total canthaxanthin (all-*trans* + *cis*) content per mg of protein showed little variation (2.20 ± 0.14 μg/mg, n = 8). This picture was confirmed by repeated comparative analysis of 4-hr-old (n = 10) and 24-hr-old (n = 10)

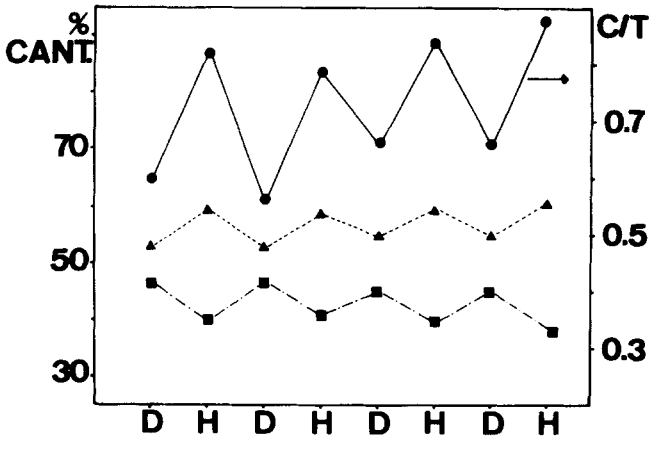


**Fig. 2.** Influence of repeated dehydration (40°C) (D) and hydration (H) (20°C) on the relative distribution of *cis*- and all-*trans*-canthaxanthin in Reference *Artemia* cysts. Upper trace (●) peak height ratios (C/T), (*cis/trans*), middle trace (▲) % *cis*, and lower trace (■) % all-*trans*-canthaxanthin in the extract.

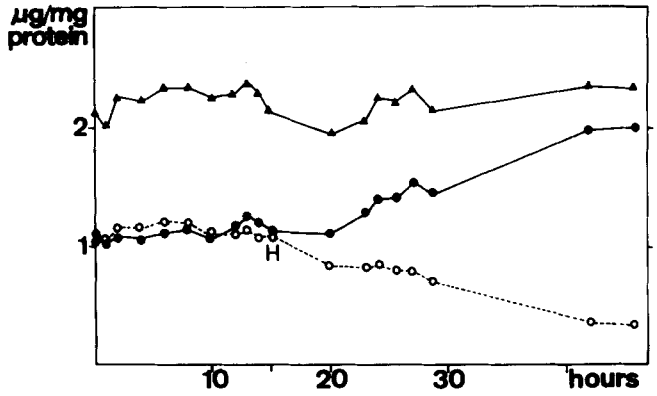


**Fig. 3.** Concentrations (μg/mg of protein) of all-*trans*-canthaxanthin (●), *cis*-canthaxanthin (○), and total canthaxanthin (▲) in the course of early *Artemia* development (SFB 522); H indicates the time of hatching.

nauplii. In the course of this 20-hr period, the all-*trans*-canthaxanthin level increased from 1.25 ± 0.05 to 1.65 ± 0.04 μg/mg, whereas the corresponding *cis*-canthaxanthin concentration dropped from 0.97 ± 0.03 to 0.69 ± 0.02 μg/mg. However, the absolute difference between the mean total canthaxanthin levels (2.23 ± 0.08 and 2.34 ± 0.06 μg/mg, respectively) was only 5%, which indicates a reasonable degree of constancy. In a separate study, starved nauplii (2–5 days old) were analyzed in duplicate experiments (duplicate results are in parentheses) using method 1. Over this 4-day period, the levels of all-*trans*-, *cis*-, and total canthaxanthin tended to approach constant values, i.e., 0.74 ± 0.05 μg/mg (0.66 ± 0.07 μg/mg), 0.15 ± 0.015 μg/mg (0.10 ± 0.01 μg/mg), and 0.89 ± 0.06 μg/mg (0.76 ± 0.07 μg/mg), respectively.

*The life cycle of* Artemia *fed on algae.* The fluctuations of the levels of all carotenoids of interest throughout the life cycle of *Artemia* fed on *Dunaliella tertiolecta* are illustrated in **Fig. 4.** In view of the near-exclusive localization of β-carotene in the intestinal tract (see below) and its occurrence in the feed, its levels probably depended on the amount of algal matter in the gut at the moment of sampling, which rationalizes the large random variations observed. However, towards the end of the life cycle, there was a consistent depletion of β-carotene parallel to an increase in canthaxanthin.

Ongoing metabolic activity was reflected by the gradual biosynthesis of echinenone from β-carotene, a well-established metabolic pathway in *Artemia* (2–4). The small amount of echinenone in starved nauplii (Fig. 1B) most likely originated from the cysts (Fig. 1A), rather than from de novo biosynthesis. In sexually undifferentiated animals, the levels of β-carotene and echinenone were significantly ($r_s$ = 0.615, $P < 0.05$, n = 12, Fig. 4) correlated. After sexual differentiation, this correlation ceased to exist ($r_s$ = 0.159, $P > 0.05$, n = 20, **Table 2,** B and D). A progressive depletion of echinenone occurred in the course

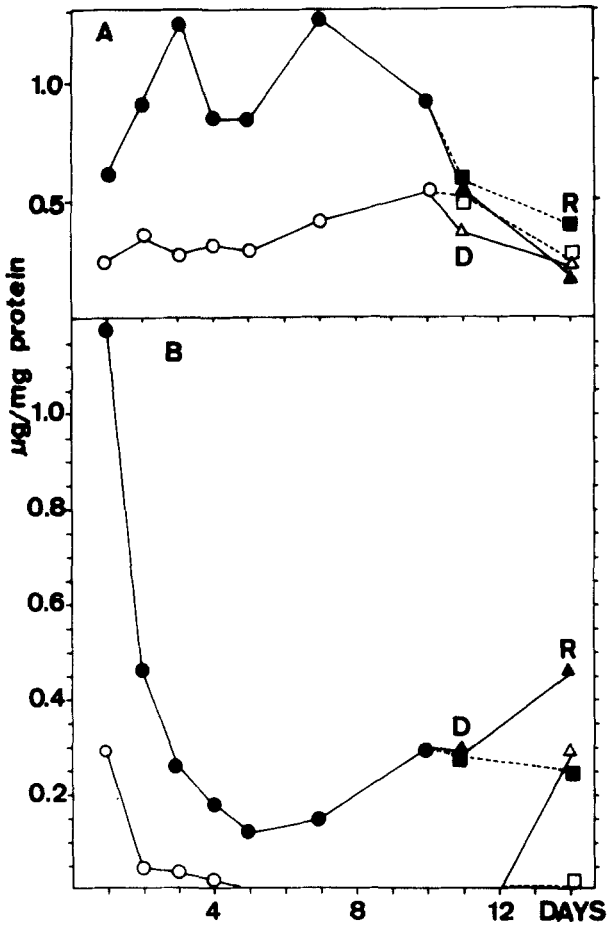


**Fig. 4.** Mean levels (n = 2) of (A) β-carotene and echinenone and (B) all-*trans*- and *cis*-canthaxanthin during the life cycle of *Artemia* (SFB) fed on *Dunaliella tertiolecta* algae. Method 2 was used for analysis. (A) β-carotene in undifferentiated animals (●), males (■) and females (▲); echinenone in undifferentiated animals (○), males (□) and females (△). (B) all-*trans*-canthaxanthin in undifferentiated animals (●), males (■) and females (▲), *cis*-canthaxanthin in undifferentiated animals (○), males (□) and females (△). D indicates the point at which differentiated immature males and females could be distinguished and separated; R indicates reproductively active animals.

of the reproductive cycle of female animals (Table 2, D). Conversely, this reduction in echinenone coincided with enhanced canthaxanthin (all-*trans* + *cis*) biosynthesis, as evidenced from the highly significant ($P < 0.01$, n = 16, Table 2, D) inverse correlation that existed between the levels of the latter and those of all-*trans*- ($r_s = -0.71$) and *cis*-($r_s = -0.78$) canthaxanthin, respectively.

Maximum canthaxanthin production was clearly situated at a relatively advanced stage (beyond stage $D_1$) of the reproductive cycle. As shown in the *footnote* to Table 2, the concentrations of echinenone and all-*trans*-canthaxanthin, unlike *cis*-canthaxanthin, in reproductively active females having oocytes in the ovaries (Table 2, $D_1$) were indeed not significantly different ($P > 0.1$) from the corresponding values in reproductively inactive females (Table 2, C). There were, however, highly significant differences between the levels of all three compounds at the early stage ($D_1$) as compared to the later stages $D_2$ and $D_3$. In contrast, the mean values at the later stages $D_2$ and $D_3$ themselves were not significantly different ($P > 0.1$) from each other. The fact that an increase in concentration of canthaxanthin was mainly observed at the onset of reproduction (Fig. 4) does, however, not preclude its synthesis before this time, although it is difficult to define exactly at what point this formation begins. In the first part of the life cycle, the all-*trans*-canthaxanthin levels steadily decreased, which suggests either that the available canthaxanthin, derived from the cysts, was further "diluted" by proteins in growing animals, or that, in any case, the formation of new canthaxanthin was still superseded by protein synthesis. In sexually differentiated immature animals, all-*trans*-canthaxanthin synthesis was clearly in progress.

However, unlike all-*trans*-canthaxanthin, *cis*-canthaxanthin rapidly became undetectable in fed *Artemia*, and remained so in pre-adults and differentiated but reproductively inactive males and females. The compound only reappeared in reproductively active females but was undetectable (Fig. 4) or negligible (Table 2, B) in males.

The same overall picture was observed for all pigments in the course of two consecutive reproductive cycles (Table 2). In this particular experiment, care was taken not to select female animals with two superimposed reproductive cycles. That is, animals at stages $D_1$ and $D_2$ of the second cycle had already deposited the offspring from the first cycle.

*Organ and tissue distribution.* **Fig. 5** represents the distribution of all-*trans*-, *cis*-canthaxanthin and their metabolic precursors in dissected organs and tissues of vitellogenic (top) and non-vitellogenic (bottom) female animals, respectively. *Cis*-canthaxanthin was particularly abundant in the oocytes and vitellogenic, as opposed to non-vitellogenic ovaries. Apart from this difference, the carotenoid distribution in both groups of animals was similar. *Cis*-canthaxanthin levels in hemolymph, although in absolute terms not impressive, significantly exceeded those of all-*trans*-canthaxanthin. All-*trans*-canthaxanthin largely predominated in the carcass and was also the only intestinal canthaxanthin. Echinenone was mainly found in the carcass and the gut and β-carotene was nearly exclusively confined to the gut.

*Subcellular localization.* Quantitative analysis of crude subcellular fractions prepared from cysts by differential centrifugation confirmed the overall picture suggested by preliminary semi-quantitative experiments (1), particularly with respect to the preferential localization of *cis*-canthaxanthin in yolk platelets and its lower abundance in the PMS (Table 1). After disruption of the yolk platelets, *cis*-canthaxanthin was previously found to be mainly

TABLE 2. Carotenoid levels in mature animals in the course of two successive reproductive cycles

| Number of Reproductive Cycle | n | Carotenoid Levels[a] (μg/mg of protein) | | | |
|---|---|---|---|---|---|
| | | All-*Trans*-Canthaxanthin | *Cis*-Canthaxanthin | Echinenone | β-Carotene |
| A. Reproductively inactive males | | | | | |
| 0 | 2 | 0.28 ± 0.02 | N.D.[b] | 0.57 ± 0.01 | 0.52 ± 0.01 |
| B. Reproductively active males | | | | | |
| 1 | 2 | 0.19 ± 0.04 | 0.02 (-) | 0.25 ± 0.06 | 0.11 ± 0.03 |
| 2 | 2 | 0.21 ± 0.04 | 0.02 ± 0.01 | 0.26 ± 0.01 | 0.13 ± 0.09 |
| Mean | 4 | 0.20 ± 0.03 | 0.02 ± 0.01 | 0.26 ± 0.03 | 0.12 ± 0.06 |
| C. Reproductively inactive females | | | | | |
| 0 | 2 | 0.28 ± 0.03 | N.D.[b] | 0.54 ± 0.07 | 0.52 ± 0.08 |
| D. Reproductively active females | | | | | |
| $D_1$ Early stage: oocytes in the ovaries | | | | | |
| 1 | 3 | 0.26 ± 0.02 | 0.06 ± 0.03 | 0.58 ± 0.05 | 0.13 ± 0.06 |
| 2[c] | 3 | 0.33 ± 0.07 | 0.08 ± 0.03 | 0.49 ± 0.09 | 0.11 ± 0.02 |
| Mean | 6 | 0.30 ± 0.06 | 0.07 ± 0.03 | 0.54 ± 0.08 | 0.12 ± 0.04 |
| $D_2$ Intermediate stage: unfertilized eggs in the oviduct | | | | | |
| 1 | 2 | 0.44 (-) | 0.24 ± 0.02 | 0.44 ± 0.05 | 0.11 ± 0.04 |
| 2[c] | 3 | 0.48 ± 0.10 | 0.27 ± 0.05 | 0.29 ± 0.05 | 0.09 ± 0.04 |
| Mean | 5 | 0.46 ± 0.07 | 0.26 ± 0.04 | 0.35 ± 0.09 | 0.10 ± 0.04 |
| $D_3$ Late stage: developing eggs in the uterus[d] | | | | | |
| 1 | 2 | 0.51 ± 0.01 | 0.29 ± 0.03 | 0.39 ± 0.04 | 0.08 ± 0.01 |
| 2 | 3 | 0.49 ± 0.03 | 0.33 ± 0.05 | 0.25 ± 0.02 | 0.10 ± 0.05 |
| Mean | 5 | 0.50 ± 0.03 | 0.31 ± 0.04 | 0.31 ± 0.08 | 0.09 ± 0.04 |

[a] Mean ± SD.
[b] Not detectable.
[c] No offspring from the first cycle remained in the uterus.
[d] No vitellogenesis in the ovaries.

deposited in the insoluble fraction, supposedly enriched in membranes (1). This phenomenon was no longer observed, which prompts a dismissal of a possible hypothesis about the compound's specific affinity for membranes. The reason for this discrepancy of results lies partly in the different extraction efficiency of the lipovitellin from yolk platelets, obtained with the modified extraction approach.

## DISCUSSION

Before the demonstration of *cis*-canthaxanthin in *Artemia* (1) nothing suggested that this organism differed from related Crustacea as far as the (nonspecific) fate of its carotenoids was concerned (2–4). Like other Crustacea, *Artemia* mobilizes carotenoids in the gonads and the eggs. However, pigmentary sexual dimorphism was supposedly nonexistent, and no specific carotenoid had been found in the female reproductive system and no metabolic changes had become apparent during development, except for cleavage of canthaxanthin-lipovitellin complexes in connection with hatching of cysts (16). The overall picture was particularly obscured by the documented claim that carotenoid-free *Artemia* retains its viability and produces viable and apparently healthy offspring (2). Yet it seems plausible that in the course of evolution *Artemia* acquired some adaptive advantages from accumulating carotenoids in its tissues and its cysts in particular. The application of a new quantitative method for the determination of both all-*trans*-, *cis*-canthaxanthin and their metabolic precursors in relation to *Artemia* development has now contributed to our insight into the possible significance of carotenoids. Because of its more characteristic biological fate it is easier to hypothesize about a possible function for *cis*- than for all-*trans*-canthaxanthin. Changes in the echinenone levels are probably less relevant but provide a useful criterion to assess overall metabolic activity. The fluctuations of β-carotene levels partly or wholly reflect food abundance and feeding activity.

The present report shows that quantitative changes in *cis*- and, to a lesser extent, all-*trans*-canthaxanthin occur in close relation to specific developmental stages. Several observations suggest that a biochemical role for *cis*-canthaxanthin, if any, is situated at an early stage of

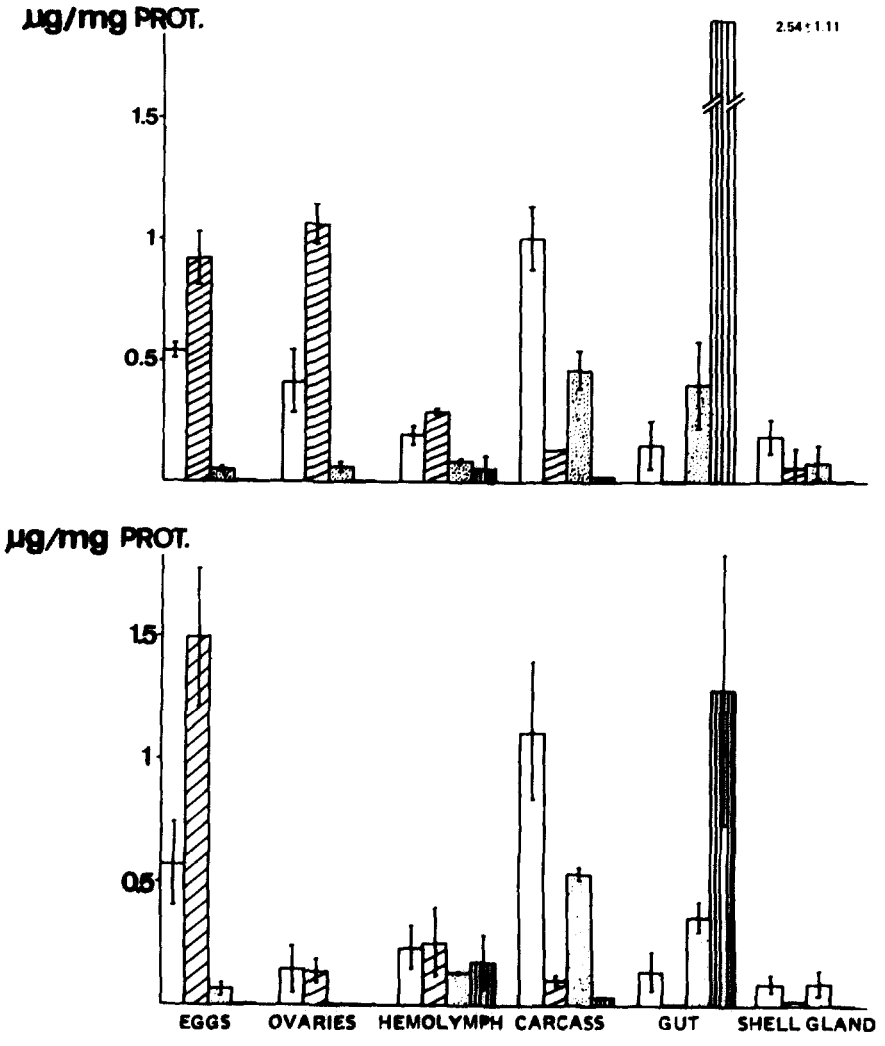


**Fig. 5.** Organ and tissue distribution of all-*trans*-canthaxanthin, *cis*-canthaxanthin, echinenone, and β-carotene in vitellogenic (top) and non-vitellogenic (bottom) female *Artemia* (SFB). Method 1 was used. For each sample, the first block (white) represents all-*trans*-canthaxanthin, the second (oblique stripes) *cis*-canthaxanthin, the third (dots) and fourth (vertical stripes) echinenone and β-carotene, respectively.

development, i.e., in cysts, before hatching. Most likely the encysted embryos are not equipped with *cis*-canthaxanthin to serve larval requirements, because the compound starts to disappear, at a fast rate, immediately after hatching. The fact that at the same time the total (all-*trans* + *cis*) canthaxanthin concentration per mg of protein remains fairly constant strongly suggests that *cis*-canthaxanthin is converted to the (already present) all-*trans*- form and not to some unknown metabolite. Since in the absence of feeding activity no new proteins are formed, the catabolism of "total" canthaxanthin apparently keeps equal pace with the disappearance of proteins, i.e., yolk consumption. *Cis*-canthaxanthin is obviously devoid of any significance in growing animals because it remains undetectable, unlike the all-*trans* form, during the greatest part of the *Artemia* life cycle. Its reappearance in reproductively active females probably only serves the function of equipping the oocytes (and, hence the cysts to be laid at a later stage) with this compound. This also rationalizes its virtual absence in males, its affinity for the female reproductive system, and its low abundance in the carcass of females, as opposed to the rather uniform distribution of all-*trans*-canthaxanthin in the body. Maximum synthesis of the *cis* as well as the all-*trans* form occurs at an advanced stage of the reproductive cycle.

The accumulation of *cis*-canthaxanthin in the yolk platelets (and in the lipovitellin) may point to a protective role. A metabolic function during pre-emergence development is less likely because, except for the *cis/trans* "cycle" in connection with dehydration/hydration, there is no real usage of either *cis*- or all-*trans*-canthaxanthin by the embryo. It has been suggested that in Crustacea carotenoprotein

formation may temporarily prevent the digestion of proteins by the embryo (17). Our data may provide indirect experimental evidence for the validity of this hypothesis in *Artemia*. It is indeed tempting to speculate that of both canthaxanthin isomers the *cis* form possesses the stereochemical conformation required to provide the "lock" on certain proteins. This lock would have to be removed after hatching to make the protein available for consumption by the nauplius. *Cis*-canthaxanthin, once dissociated from the protein, would then spontaneously be converted to the more stable all-*trans* form. The gradual disappearance of *cis*-canthaxanthin observed in nauplii would thus reflect the progressive deblocking of the yolk protein in the course of its digestion by the animal.

However, at this point there is no experimental evidence indicating that the association of lipovitellin with *cis*-canthaxanthin indeed protects it from proteolytic breakdown. How the reversible *cis/trans* conversion in cysts may fit into this picture remains unclear as well.

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