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# Carotenoids of two freshwater amphipod species (*Gammarus pulex* and *G. roeseli*) and their common acanthocephalan parasite *Polymorphus minutus*

Maria Gaillard, Cédrik Juillet, Frank Cézilly, Marie-Jeanne Perrot-Minnot*

*Equipe Ecologie Evolutive, UMR CNRS 5561 Biogéosciences, Université de Bourgogne, 6 Boulevard Gabriel, Dijon, France*

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**Abstract**

Carotenoid compositions of two freshwater *Gammarus* species (Crustacea: Amphipoda) and of their common acanthocephalan parasite *Polymorphus minutus* were characterized. The effect of carotenoid uptake by the parasite was addressed by comparing the carotenoid content of uninfected and infected female hosts. Using high-pressure liquid chromatography (HPLC), co-chromatography of reference pigments and electron ionization mass spectrometry of collected HPLC fractions (EI-MS), several xanthophylls and non-polar compounds were identified. Seven kinds of carotenoids, mainly xanthophylls, were identified in gammarids. Astaxanthin was predominant, amounting to 40 wt.% of total carotenoid in both uninfected *G. pulex* and *G. roeseli.* By contrast, we found only non-polar compounds with a predominance of esterified forms of astaxanthin in *P. minutus* larvae. No significant effect of infection on carotenoid content was evidenced in *G. pulex* and *G. roeseli* females. Our study highlights the use of a Matrix Solid Phase Dispersion as an efficient extraction method of both xanthophylls and non-polar pigments in small samples, including lipid-rich ones as *P. minutus* parasite. We discuss on the presumptive pathway leading to the formation of free astaxanthin in gammarids via hydroxy compounds, and on the accumulation of esters of astaxanthin in parasites.


*Keywords:* Acanthocephala; Astaxanthin ester; Carotenoid; Freshwater crustacea; *Gammarus*; HPLC; EI-MS

## 1. Introduction

Over the last 10 years, a growing interest for the adaptive significance of carotenoid-based colorations in animals has developed in both evolutionary biology and behavioural ecology (Lozano, 1994; Olson and Owens, 1998; Faivre et al., 2003). An important aspect of the evolution of carotenoid-based colorations is that animals cannot synthesize carotenoids de novo and must therefore obtain them from their food. Herbivores generally obtain more carotenoid per unit mass of food than do carnivores, with omnivores falling in between (Olson and Owens, 1998). However, the ability to use the carotenoids present in food depends largely on the genotype and physiology of organisms. Environmental agents, acting independently of diet, can directly interfere with carotenoid storage. For instance, it has been shown that coccidia, an intestinal parasite, interfere with the absorption of carotenoids in chickens (Ruff et al., 1974). More recently, retinol uptake by the nematode *Onchocerca volvulus* in vivo and by *Plasmodium falciparum* in vitro have been shown to play a role in host vitamin A depletion and associated pathology (Bradley et al., 2001; Mizuno et al., 2003). Little is known however of the impact of macroparasites on the carotenoid content and composition of their hosts.

Host–parasite interactions involving amphipod (Crustacea) hosts and acanthocephalan parasites constitute an ideal model to investigate the consequences of carotenoid uptake by parasites on the carotenoid content and composition of their hosts. First, as first consumers, detritus feeders and scavengers, amphipods recycle, metab-

* Corresponding author. Tel.: +33 3 80 39 63 40; fax: +33 3 80 39 62 31.
*E-mail address:* mjperrot@u-bourgogne.fr (M.-J. Perrot-Minnot).

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olize and concentrate carotenoids found in their food, which include algae, decaying vegetation and small invertebrates (MacNeil et al., 1997). Second, amphipods are intermediate hosts for numerous acanthocephalan species, several of which show carotenoid-based colorations at both the larval and adult stages (Barrett and Butterworth, 1968, 1973). Parasites develop inside their intermediate hosts and therefore must obtain carotenoids from them. However, the consequences of infection with acanthocephalans on the carotenoid content and composition of amphipods remains undocumented.

To obtain such information, refined techniques of carotenoid extraction, separation, and identification are needed. Acanthocephalans have lipid-rich tissues very resistant to disruption. Previous studies of the carotenoid content of Acanthocephalans and of one of their intermediate hosts, *Gammarus pulex*, relied on successive steps including open-columns and thin layer chromatography techniques (Barrett and Butterworth, 1968, 1973). These procedures were time and solvent consuming, required large amounts of fresh material, and were of limited efficiency for carotenoid quantification. Advanced techniques of extraction and separation have now replaced these original procedures, particularly high-pressure liquid chromatography (HPLC), which considerably enhance assay speed and resolution.

Here, we present results about the impact of infection with the acanthocephalan *Polymorphus minutus* on the carotenoid content and composition of two freshwater amphipods, *G. pulex* and *Gammarus roeseli*. We used Matrix Solid Phase Dispersion (MSPD) procedure (Barker, 2000) to perform complete carotenoid extraction from these two amphipod species, and from larvae of their common acanthocephalan parasite *P. minutus*. Carotenoids were identified by means of reversed-phase liquid chromatography (RP-HPLC) with a C30 bonded phase, co-chromatography of reference pigments, and electron ionization mass spectrometry of collected HPLC fractions (EI-MS).

## 2. Materials and methods

### 2.1. Chemicals

Carotenoid standards (astaxanthin, lutein, zeaxanthin, canthaxanthin, β-carotene and lycopene) were purchased from Extrasynthèse (Genay, France). HPLC-grade solvents (Prolabo) containing 0.01% of 2,6-di-*tert*-butyl-*p*-cresol (BHT; Fluka Chemika, Switzerland) were used for extraction procedures and chromatographic analyses. BHT acts as an antioxidant and prevents degradation of carotenoids.

### 2.2. Collection of samples

Females of *G. pulex* and *G. roeseli* were collected in April and May 2003 in River Ouche (Dijon, Eastern France) and River Albane (Trochères, Eastern France), respectively. Both uninfected females and females infected with *P. minutus* parasite were collected and analyzed simultaneously. As all acanthocephalan parasites, *P. minutus* develop in their intermediate host up to a last larval stage, the cystacanth, which has accumulated nutrients from its hosts (Crompton and Nickol, 1985; Taraschewski, 2000). In the field, infected gammarids were easily spotted from the presence of an orange dot that corresponds to the cystacanth, visible through the translucent cuticle of the host. Following collection, females were starved for 3 days in continuously oxygenated fresh water taken from the river. Eggs were removed from the brood pouch of uninfected females and counted. No eggs were found in parasitized females, in agreement with previous studies reporting complete castration of *G. pulex* and *G. roeseli* by *P. minutus* (Ward, 1986; Bollache et al., 2002; Juillet, 2003). Individual females were dried on an absorbant tissue, weighed on a semi-analytical balance with a 0.01-mg accuracy, and dissected. The infected females analyzed harbored only one cystacanth, and were thus weighed with their parasite. In fact, *P. minutus* cystacanth dry mass ($0.11 \pm 0.01$ mg; Barrett and Butterworth, 1971) was representing less than 7.5% females mass. Dissection was processed quickly on an aluminium foil, thereafter folded in two and frozen at −80 °C, to avoid losing haemolymph and to minimize carotenoid degradation. Freshly laid eggs, characterized by a uniform brownish pigmentation, were collected from the brood pouch of uninfected females and pooled by 50 for carotenoid analysis. Cystacanths from parasitized females were rinsed in deionized water, and frozen at −80 °C in an aluminium foil folded in two. Based on preliminary carotenoid content analysis, we pooled 15 to 20 cystacanths to extract enough carotenoids for the identification of the fractions separated by HPLC. Samples of *P. minutus* from each population and host species were pooled separately.

### 2.3. Extraction of carotenoids

Matrix Solid Phase Dispersion (MSPD) is particularly convenient for the extraction of carotenoids in biological tissues (Barker, 2000; Dachtler et al., 2001). Since carotenoids are light- and oxygen-sensitive, their processing has to be gentle and rapid prior HPLC analysis. MSPD procedure was thus chosen to minimize risks of degradation of pigments or formation of artefacts during their isolation. By the combination of polar and non-polar solvents in MSPD procedure, we achieved a complete extraction of carotenoids including xanthophylls and carotenes.

The disruption of sample tissues is a critical step for successful MSPD. It was achieved by crushing on dry ice each sample frozen in aluminium foil, using a steel pestle pre-frozen at −80°C, until a fine homogenous powder was obtained. Two hundred milligrams (parasites) or 400 mg (gammarids) of Isolute MSPD grade C18 sorbent material

(International Sorbent Technology, UK) were added and well mixed on dry ice, and filled into an empty MSPD column. A frit was placed on the top of the powder and a compact column bed was created by compressing the sample with the frit inserter. To allow interaction between the sample matrix and the C18 phase, the column was conditionized with 10 ml of deionized water. Hundred microliters (parasites) or 500 μl (gammarids) of internal standard (2 ng/μl of lycopene in 50% methyl-*tert*-butyl ether (MTBE)/50% methanol) were applied on the column. Then, pigments were eluted using successively 500 μl (parasites) or 1000 μl (gammarids) of methanol, and 500 μl (parasites) or 1000 μl (gammarids) of MTBE. The extracts were completely evaporated under nitrogen in a dark room, and the carotenoids were redissolved in 50 μl (parasites) or 200 μl (gammarids) of 50% MTBE/50% methanol just before the injection into HPLC column.

### 2.4. HPLC analysis

HPLC separations were performed on a 250×4.6 mm stainless steel ProntoSIL C30 reversed-phase column (3-μm particle size and 200-Å average pore diameter; Bischoff, Leonberg, Germany). Chromatographic analyses were conducted on a Waters system (Milford, MA, USA) controlled by the Millenium$^{32}$ software. Fifty microliters of each sample were injected into a 717 Plus Autosampler. Separations of carotenoid extracts were carried out using a mixture of MTBE, methanol and deionized water as mobile phase, delivered by a 600 E Multisolvent pump at a flow rate of 1 ml/min. An isocratic mixture of methanol/MTBE/water (86:10:4 v/v/v) were used for 10 min, followed by a 40-min linear mobile phase gradient from 86:10:4 to 10:90:0 methanol/MTBE/water v/v/v. Between two injections, the column was returned to the initial conditions by applying a 20-min gradient back to initial conditions, plus 10 min of column conditioning. These conditions allowed a complete elution of pigments from xanthophylls to non-polar carotenoids in one step. Absorption spectra were collected from 250 to 600 nm with a Waters 996 photodiode array detector. Chromatograms were plotted at 450 nm, and peak identification was effected by comparing elution order, retention times and absorption properties ($\lambda_{max}$ values) with pure standards.

We established separate calibration curves for astaxanthin, zeaxanthin, lutein, β-carotene and lycopene, by injecting six increasing doses of pure standard. Peak area for these carotenoids could thus be directly converted into quantities. Quantities of other carotenoids and total carotenoid content were estimated by calculating the mean of quantities derived from astaxanthin, zeaxanthin, and lutein calibration curves. The extraction output was calculated for each sample, as the ratio of the estimated final quantity of lycopene to the initial quantity included as internal standard. Data are analyzed and presented after correction for extraction output.

### 2.5. Saponification

Saponification was conducted on dry parasite carotenoid extract with 500 μl of a methanolic 0.5% NaOH solution. The process was conducted at room temperature in darkness under nitrogen stream, until the reaction mixture was completely evaporated (within 15 min). Thereafter, the saponified sample was immediately resuspended and submitted to HPLC.

### 2.6. HPLC-MS coupling

A HP 1050 HPLC system (Hewlett Packard) was coupled with an Esquire-LC ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) for the HPLC-MS experiments. The chromatographic runs were operated in the same conditions as described in Section 2.4. HPLC eluent was split with a split flow of 200 μl/min to a Gilson 118 UV/Visible absorbance detector ($\lambda$=450 nm) and to the mass spectrometer with a split flow of 800 μl/min. Molecules were ionized in an atmospheric pressure chemical ionization (APCI) source in the negative mode. The corona needle voltage was −2000 V and the ion trap scanned from 50 to 1200 m/z every 350 ms.

## 3. Results

A variety of carotenoids were evidenced in both gammarid species (Table 1). From a qualitative point of view, the HPLC profile indicated a comparable carotenoid content for the different extracts within each species, as well as between the two *Gammarus* species (Fig. 1a,b). HPLC traces of *G. pulex* and of *G. roeseli* revealed six well-separated fractions, corresponding to xanthophyll compounds (Table 1, Fig. 1a,b). The comparison of retention time and absorption spectra to authentic carotenoids allowed the characterization of most of these compounds. The identification of xanthophyll pigments was further con-

Table 1
Spectral and mass spectrometry characteristics of carotenoids from Acanthocephalan parasites infecting *G. pulex*

| HPLC range | Carotenoid | λ max | Ions m/z |
|---|---|---|---|
| 1 | Astaxanthin | 470 nm | 596 |
| 2 | β,β-carotene-3,4,3′ -triol | (424), 447, 476 nm | 582 |
| 3 | Lutein | (420), 444, 473 nm | 568 |
| 4 | Zeaxanthin | (425), 452, 479 nm | 568 |
| 5 | Unidentified compound | (425), 453, 480 nm | 566 |
| 6 | β-cryptoxanthin | (425), 453, 481 nm | 552 |
| 7 | β-carotene | 452, 479 nm | |
| 8 | Esterified astaxanthin | 479 nm | |
| 9 | Unidentified non-polar compounds | | |

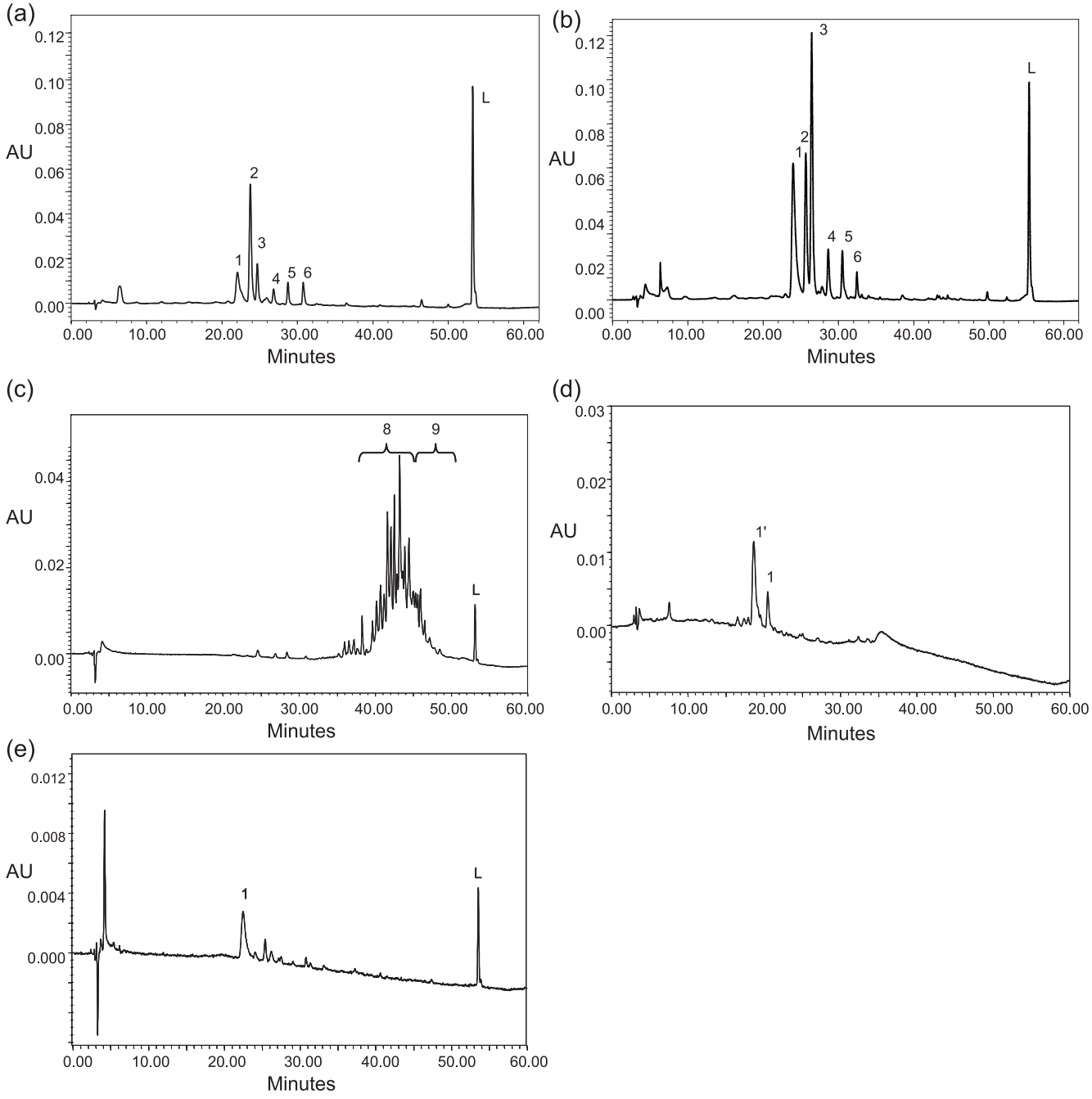


Fig. 1. HPLC carotenoid profile of (a) a *G. pulex* uninfected female, (b) a *G. roeseli* uninfected female, (c) the acanthocephalan parasite *P. minutus* and (d) a saponified carotenoid extract of *P. minutus*, (e) freshly laid eggs of *G. pulex*. Peak identification is given in Table 1; L, internal standard (lycopene), 1′, astacene. For HPLC conditions, see Section 2.

firmed by mass spectrometry (Table 1). The UV spectrum of the fraction 1 showed a symmetrical curve with an absorption maxima at 470 nm and the mass spectrum a peak at *m/z* 596. These characteristics are typical of astaxanthin and we confirmed the occurrence of astaxanthin with chromatography of an authentic standard. The fraction 2 showed two absorption maxima at 447 and 476 nm and a shoulder at 424 nm which was consistent with the presence of a β,β-carotene type chromophore. Based on a negative ionization base peak at *m/z* 582 and on elution order by HPLC, it was assumed that this fraction could be β,β-carotene-3,4,3′ -triol (Tsushima et al., 1989; Ohkudo et al., 1999). The fractions 3 and 4 were respectively identified as lutein and zeaxanthin, based on absorption maxima and mass spectrum of these fractions and of their corresponding pure standards (Table 1). Fraction 6 was identified as β-cryptoxanthin, by comparison of the absorption maxima and mass spectrum to the published data (Table 1). Fraction 5 could not be confidently identified. Free astaxanthin was the major carotenoid detected in freshly laid embryos (Fig. 1e).

Quantification of carotenoid was performed after correcting for extraction output using lycopene as internal standard, and on the basis of calibration curves. Extraction output averaged 51% (±19). On average, astaxanthin made up 40% of total carotenoid content, in weight, of uninfected females of *G. pulex* and *G. roeseli* (min 21%–max 51%). The second major carotenoid was β,β-carotene-3,4,3′ -triol, representing about one quarter of total carotenoids (Table 2). Lutein was present at less than 20%, and the other three carotenoids, β-cryptoxanthin, zeaxanthin, and the unidentified one, at less than 10% of total carotenoids (Table 2). β-carotene was detected in very small quantities in some samples. No other non-polar compounds were found. No significant quantitative difference in carotenoid content was found between the two gammarid species (ANOVA on total carotenoid content between uninfected *G. pulex* and *G. roeseli*, $F_{11}$=0.28, $P$>0.05; Table 2).

HPLC traces of *P. minutus* appeared to contain exclusively a diversity of non-polar forms, which were not well separated by HPLC (Fig. 1c). Several peaks (fraction 8 on Fig. 1c) showed a UV symetrical profile with an absorption maxima at 479 nm, and did not provide any ions with mass spectrometry. These data strongly suggest the predominance of several forms of esterified astaxanthin in *P. minutus* cystacanths, differing by the type of fatty acid associated to astaxanthin. Few minor unidentified forms (fraction 9, Fig. 1c) presented UV spectrophotograms with a vibrational fine structure ($\lambda_{max}$ comprised between 450 and 453 nm). The presence of esterified forms of astaxanthin was confirmed by saponification of an extract, yielding only astaxanthin and its oxidized derivative, astacene (Fig. 1d).

No qualitative difference was observed between uninfected and *P. minutus*-infected females in both *Gammarus* species (Permutation tests on each carotenoid proportion, $P$>0.05 in all tests; Table 2). No quantitative changes were evidenced either (ANOVA on total carotenoid content between uninfected and infected *G. pulex*, $F_8$=3.51, $P$=0.087, and between uninfected and infected *G. roeseli*, $F_{13}$=1.73, $P$=0.19), despite a tendency for infected *G. pulex* females to have more carotenoids and relatively less astaxanthin compared to uninfected ones (Table 2).

## 4. Discussion

The Matrix Solid Phase Dispersion (MSPD) method allowed us to achieve a complete isolation of xanthophylls and non-polar compounds, in both *Gammarus* species and their parasite. Carotenoid extraction was considerably improved compared to classical liquid–liquid or solid-phase procedures (Dachtler et al., 2001). Whole organisms, including solid parts (cuticle) and viscous or liquid parts (tissues, haemopymph), could be directly mixed with the sorbent material, thus minimizing loss or degradation. Through coupling HPLC with mass spectrometry, several carotenoids were unambiguously identified, such as a form of carotenoid not frequently reported so far, β,β-carotene-3, 4, 3′ -triol (Tsushima et al., 1989; Ohkudo et al., 1999). In addition, the higher resolution in phase separation achieved by the use of a C30 column instead of C18 allowed clear separation and unambiguous identification of most carotenoids, including esterified forms. Preliminary experiments using C18 column without MTBE failed to discriminate lutein from zeaxanthin, and to elute non-polar compounds from the column.

Astaxanthin was the predominant carotenoid in both *Gammarus* species (more than 40% in uninfected females), as in other amphipod species (Czeczuga, 1980a; Dembitsky and Rezanka, 1996), in crustaceans (Goodwin, 1960), and more generally in marine animal tissues. Astaxanthin has been shown to be a powerful quencher of singlet oxygen activity and a strong scavenger of oxygen free radicals. Its antioxidant properties are at least ten times stronger than that of vitamin A, and a hundred times stronger than vitamin E (Mike, 1991). Free astaxanthin is the predominant carotenoid provided in eggs of decapods (several references in Sagi et al., 1995 and in Berticat et al., 2000), and in *G. pulex* (present study), and *G. roeseli* (data not shown), where it may protects vitellin against oxidation. Astaxanthin accumulates in the aquatic food chain and its good bioavailability has been shown in fishes, and in prawn and lobster (several references in Petit et al., 1998).

The second predominant carotenoid was β,β-carotene-3,4,3′ -triol (Tsushima et al., 1989; Ohkudo et al., 1999). Its role in carotenoid metabolism is not well established yet.

Table 2
Carotenoid composition in *G. pulex* and *G. roeseli* females, uninfected or infected with *P. minutus* acanthocephalan parasite

| Carotenoids | Carotenoid composition μg/g (fresh weight) (%) | | | |
|---|---|---|---|---|
| | *G. pulex* uninfected ($n$=5) | *G. pulex* infected ($n$=4) | *G. roeseli* uninfected ($n$=7) | *G. roeseli* infected ($n$=7) |
| Astaxanthin[a] | 88.4±62.6 (43.1) | 118.2±69 (30.9) | 100.3±41.1 (43.7) | 78.7±19.5 (46.3) |
| β,β-carotene-3,4, 3′ -triol[b] | 54.6±37 (24.5) | 138.3±87.2 (34.7) | 61±28.3 (25.3) | 45.1±24 (23.9) |
| Lutein[a] | 21.6±10.2 (13.8) | 38.3±17.4 (10.3) | 44.5± 25.2 (17.9) | 35.8±16.6 (19.6) |
| Zeaxanthin[a] | 11.7±5.1 (7.8) | 25±5.9 (7) | 13.7±10.4 (5.2) | 6.5±4 (3.4) |
| Unidentified compound[b] | 13.5±11.8 (5.4) | 36.6±6.4 (10.7) | 14.9±8.1 (6.1) | 8.9±7.6 (4.5) |
| β-cryptoxanthin[b] | 13.4±11 (5.5) | 23.1±7.9 (6.3) | 4±1.7 (1.7) | 4.2±2.6 (2.3) |
| β-carotene | – | – | – | – |
| Total[b] | 203.1±130.2 | 379.5±152.9 | 252.4±105.5 | 188.7±71.3 |

[a] Direct quantification from corresponding authentic standard.
[b] Estimates (see Section 2).

Lutein, zeaxanthin, and β-cryptoxanthin were also detected in both gammarid species. One compound could not be confidently identified (fraction 5). Its mass spectrum showed a peak at *m/z* 565.6, similar to hydroxyechinenone (Yokoyama and Miki, 1995). However, its absorption spectra showed two maxima and a shoulder, more compatible with a reduced form of hydroxyechinenone (Schwartzel and Cooney, 1972). Very low levels of β-carotene were found in some individuals of both *Gammarus* species. These small amounts of β-carotene could be due to its rapid metabolisation prior to carotenoid analysis. In fact, we have indirect evidence for β-carotene uptake by *G. pulex*, since this carotenoid is stored in at least one species of its acanthocephalan parasites (Cezilly et al., in prep.). Alternatively, β-carotene could represent a minor carotenoid fraction in the diet of both gammarid species, and its slow metabolic rate could make it still available for parasite uptake. No detectable levels of β-doradexanthin nor of echinenone and canthaxanthin were found. The carotenoid content of *G. pulex* reported here is partly in agreement with the study of Barrett and Butterworth (1968), where lutein and astaxanthin were predominant. However, we did not found the two other carotenoids isolated by these authors: canthaxanthin and a carotenoid tentatively identified as xanthophyll epoxide. Instead, we found four other pigments, two of which are commonly found in aquatic organisms, zeaxanthin and β-cryptoxanthin. The predominance of astaxanthin in uninfected *G. pulex* and *G. roeseli* suggests the transformation of dietary carotenoids, in particular β-carotene, into free astaxanthin, a process that has been shown in most crustaceans (Nègres-Sadargues, 1975; Czeczuga, 1980b). Some intermediate products of the metabolic pathway from lutein to astaxanthin (α-doradexanthin, β-doradexanthin) (Czeczuga, 1980b), and from β-carotene to astaxanthin (echinenone and canthaxanthin) are commonly found in crustaceans (Davies et al., 1970; Nègres-Sadargues, 1975), and in gammarids in particular (Czeczuga, 1980b), but were not detected in the present study. However, several other intermediate products from the conversion of β-carotene to astaxanthin were found, that allow us to propose a biosynthetic pathway to astaxanthin. The occurrence of β-cryptoxanthin and zeaxanthin suggests that biosynthesis pathway starts with two hydroxylation steps (Fig. 2), rather than two oxidation steps in the echinenone-canthaxanthin pathway (Yokoyama and Miki, 1995). β,β-Carotene-3,4,3′-triol could be a third intermediate hydroxy compound, originated from the hydroxylation of zeaxanthin, and subsequently metabolized into astaxanthin (Fig. 2). If further analysis identifies fraction 5 as reduced hydroxyechinenone, this compound could also be an alternative intermediate in the production of β,β-carotene-3,4,3′-triol from β-cryptoxanthin. Finally, astaxanthin would originated from β,β-carotene-3,4,3′-triol by oxidation reactions. In these presumptive pathways, hydroxy-carotenoids are the main intermediate compounds in the formation of astaxanthin, suggesting that the introduction of a ketogroup is not a direct reaction, but rather results from the oxidation of hydroxy compounds, as

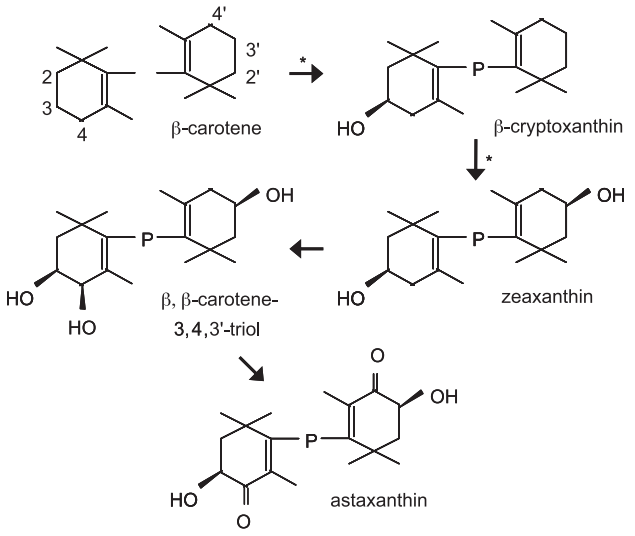


Fig. 2. Carotenoids encountered in the amphipods *G. pulex* and *G. roeseli* and their possible biosynthetic relationship. Asterisk after the reactions reported by Yokoyama and Miki (1995).

proposed by Davies et al. (1970). Although ketocarotenoids are essential for astaxanthin formation, it is possible that (terminal) oxidative reactions leading to the formation of astaxanthin were too transient in the studied organism to yield detectable levels of these middle metabolism products.

In the aquatic bacteria *Agrobacterium aurantiacum*, the two alternative pathways—the echinenone-canthaxanthin one, and the β-cryptoxanthin-zeaxanthin one—have been shown to produced astaxanthin. The order of the hydroxylation and oxydation reactions, determining the pathway used, was controlled by the culture condition, and more precisely by the amount of oxygen (Yokoyama and Miki, 1995). To our knowledge, no study has addressed the changes in carotenoid content in aquatic crustaceans consecutive to environmentally induced shifts in biosynthesis pathways for the formation of astaxanthin, despite their high sensitivity to water oxygenation. Alternatively, the lacking of ketocarotenoids as intermediate products of astaxanthin synthesis in the species studied, compared to previous studies in other gammarid species (Barrett and Butterworth, 1968; Czeczuga, 1980a), could be due to interspecific genetic variability in the enzymatic capabilities and different dietary habits.

The total carotenoid content herein reported in *G. pulex* and in *G. roeseli* is 20–100 times higher than in previous studies on another *Gammarus* species, *G. lacustris* (Czeczuga, 1980a), but close to estimates from prawn (150–200 μg/g fresh weight; Petit et al., 1998). Assuming a body water content in crustacean of 70% (Vonk, 1960), *G. pulex* and *G. roeseli* would contend on average 676 and 794 μg/g dw respectively, which is within the range of total carotenoid content reported for instance in *Daphnia magna* (800 μg/g dw; Borgeraas and Hessen, 2002) or in two copepod species (around 870 μg/g dw; Lotocka et al., 2004). This discrepancy in estimates of total carotenoid

content in *Gammarus* species could result from a better quantification of total carotenoids through our extraction and injection procedures, particularly by limiting loss and degradation of carotenoids and correcting for extraction–injection output.

The carotenoid composition of *P. minutus* is clearly contrasting with that of its hosts. We found only esterified astaxanthin in cystacanths of *P. minutus*, as previously reported by Barrett and Butterworth (1968). Although the HPLC technique is inappropriate for the quantification of esterified carotenoids, the extraction yield and resolution in phase separation allowed unambiguous identification of several forms of esterified astaxanthin. An enzymatic hydrolysis step was included in the protocole to yield all free astaxanthin from *P. minutus* cystacanths in some extracts. Since no esterified form of astaxanthin was found in the *Gammarus* hosts, it is likely that esterification is occurring in the process of astaxanthin storage by the parasite. The cystacanth of *P. minutus* is storing large quantities of wax esters as a reserve energy store, which amount 90% of the total lipids (Barrett and Butterworth, 1971). Fatty acid (FA) composition of astaxanthin esters in *P. minutus* includes mainly long chain C18:1 FA (Barrett and Butterworth, 1971), which may result from the elongation of FA taken from the host (Taraschewski, 2000). Whether the uptake of carotenoid by *P. minutus* cystacanth is exclusively restricted to astaxanthin or not is not known, but the synthesis of astaxanthin de novo is more speculative than simply assuming selective uptake as the sole source of astaxanthin. Astaxanthin esters, especially with C18:1 FA, function as powerful antioxidant agents under both hydrophobic and hydrophilic conditions (Kobayashi and Sakamoto, 1999). The adaptive significance of carotenoid storage in *P. minutus* under several forms of esterified astaxanthin might be related to protection against oxidative stress, especially lipid peroxidation.

The lack of significant effect of carotenoid uptake by *P. minutus* larvae on its hosts *G. pulex* and *G. roeseli*, herein reported, certainly deserves more investigation. The increasing trend of carotenoid content (in particular astaxanthin) in infected *G. pulex* females could be attributable to the fact that reproductive females provision eggs with carotenoids via the vitellin, a lipo-carotenoprotein of yolk in crustacean eggs (Chang et al., 1993). Astaxanthin was the only carotenoid found in young embryos of *G. pulex*, as reported also in other crustaceans (Sagi et al., 1995; Berticat et al., 2000). Since the parasite *P. minutus* induce complete castration of females (Ward, 1986; Bollache et al., 2002), infected females could accumulate more carotenoids compared to uninfected ones, especially if the latter were analyzed just after egg laying (and eggs are removed), as it was the case in the present study.

This tendency should be reassessed based on a larger sample and on both sexes, by mean of spectrophotometry. Such analysis will be facilitated by the quick and reliable MSPD extraction method presented here.

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