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Interspecific differences in carotenoid content and sensitivity to UVB radiation in three acanthocephalan parasites exploiting a common intermediate host

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Few endoparasite species are pigmented. Acanthocephalans are an exception however, with several species being characterised by yellow to orange colouration both at the immature (cystacanth) and adult stages. However, the functional and adaptive significance of carotenoid-based colourations in acanthocephalans remains unclear. One possibility is that the carotenoid content of acanthocephalan cystacanths acts as a protective device against ultra-violet radiation (UVR) passing through the translucent cuticle of their crustacean hosts. Indeed, acanthocephalans often bring about behavioural changes in their aquatic intermediate hosts that can increase their exposure to light. Carotenoid composition and damage due to ultra-violet – B (UVB) radiation were investigated in three acanthocephalan parasite species that induce contrasting behavioural alterations in their common intermediate host, the crustacean amphipod *Gammarus pulex*. The fish acanthocephalans *Pomphorhynchus laevis* and *Pomphorhynchus tereticollis* both induce a positive phototaxis in gammarids, such that infected hosts spend more time out of shelters, while remaining benthic. The bird acanthocephalan *Polymorphus minutus*, on the other hand, induces a negative geotaxis, such that infected hosts typically swim close to the water surface, becoming more exposed to UV radiation. We show that differences in cystacanth colouration between acanthocephalan species directly reflect important differences in carotenoid content. The two fish parasites exhibit a contrasting pattern, with *P. tereticollis* harbouring a large diversity of carotenoid pigments, whereas *P. laevis* is characterised by a lower carotenoid content consisting mainly of lutein and astaxanthin. The highest carotenoid content is found in the bright orange *P. minutus*, with a predominance of esterified forms of astaxanthin. Exposure to UVB radiation revealed a higher susceptibility in *P. laevis* larvae compared with *P. tereticollis* and *P. minutus*, in terms of sublethality (decreased evagination rate) and of damage to DNA (increased cyclobutane pyrimidine dimers production). Although we found important and correlated interspecific differences in carotenoid composition and tolerance to high UVB radiation, our results do not fully support the hypothesis of adaptive carotenoid-based colourations in relation to UV protection. An alternative scenario for the evolution of carotenoid accumulation in acanthocephalan parasites is discussed.

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1. Introduction

Ever since the work of Alfred Russell Wallace (1905), the adaptive value of animal colours has stimulated much empirical and theoretical work (Cott, 1940; Endler, 1990; Lozano, 1994; Bradbury and Verhencamp, 1998). Little attention, however, has been paid to the colouration of parasites. This may stem from the fact that a large number of parasites live inside their hosts, have reduced sense organs, and therefore do not seem to benefit from possessing photoreflecting pigmentation. One noticeable exception, however, is found among acanthocephalan parasites, where several species show bright yellow to orange colouration, particularly at the cystacanth larval stage (Crompton and Nickol, 1985). Orange colouration in animals is generally due to the presence of carotenoids. Animals are unable to synthesise carotenoids de novo and, therefore, must obtain them from their food (Lozano, 1994; Lesser, 2006). Previous studies (Van Cleave and Rausch, 1950; Barrett and Butterworth, 1968, 1973; Ravindranathan and Nadakal, 1971; Gaillard et al., 2004; Duclos, 1996. Functional significance of pigment in larval *Corynosoma constrictum* Van Cleave, 1918 (Acanthocephala: Polymorphidae). Ph.D. Dissertation, University of Nebraska, USA) have indeed shown that the yellow to bright orange colouration of a number of acanthocephalan parasites depends upon carotenoid pigments that they obtain from their hosts. Although carotenoids may not be essential for the adult acanthocephalan parasite in the intestine of their vertebrate final host (Barrett and Butterworth, 1968), their role and function in cystacanths infecting crustacean intermediate hosts is of evolutionary interest (Moore, 2002).

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Acanthocephalan parasites have complex life cycles, and typically exploit arthropods as intermediate hosts. A large proportion of acanthocephalan parasites with coloured cystacanths (the stage at which the parasite becomes infective to its definitive host) exploit amphipods as intermediate hosts, and in most cases the coloured cystacanths are visible through the host's translucid cuticle. Two adaptive functions have been proposed for the presence of carotenoid-based colourations in acanthocephalan cystacanths (Bakker et al., 1997; Moore, 2002). It was initially proposed that the colouration of cystacanths may act to enhance trophic transmission of the parasite to its final host, through increased conspicuousness of infected intermediate hosts (Bakker et al., 1997). However, a recent study (Kaldonski et al., 2009) indicates that the orange colouration of fish acanthocephalans actually plays no role in the increased vulnerability of infected gammarids to predation by fish. In addition, Bethel and Holmes (1977) pointed out that cystacanths of *Polymorphus minutus*, an acanthocephalan species using amphipods as intermediate hosts and wildfowl as definitive hosts, are bright orange, although ducks do not rely on vision to capture their crustacean prey. Another adaptive explanation relies on the well known function of carotenoids in photoprotection, either as reactive-oxygen species (ROS) scavengers or as ultra-violet (UV)-screening compounds (Cockell and Knowland, 1999; Stahl and Sies, 2003), particularly in aquatic species (Lamare and Hoffman, 2004; Hansson and Hylander, 2009). Among variable selection pressures, carotenoid accumulation may therefore be interpreted as an adaptation to UV threat, in particular in zooplankton (see Hansson and Hylander, 2009 for a review). Although a photoprotective function of carotenoids in pigmented acanthocephalans has been hypothesised (Barrett and Butterworth, 1973; Bakker et al., 1997), direct evidence is lacking. However, this hypothesis might be particularly relevant for acanthocephalan species that, through manipulating their host behaviour to make them more vulnerable to predation by appropriate definitive hosts (Cézilly et al., 2000; Moore, 2002), become exposed to more UV radiation. Indeed, acanthocephalan species with yellow-orange cystacanths tend to exploit intermediate hosts with translucid cuticles such as crustacean amphipods whereas, noticeably, white species of acanthocephalan generally occur in intermediate hosts with dark opaque cuticle (such as *Moniliformis moniliformis* in cockroaches, *Plagiorhynchus cylindraceus* in woodlice or *Acanthocephalus anguillae* in asellids). UVB radiation may penetrate several metres in clear water (Smith et al., 1992) and, therefore, acanthocephalan cystacanths infecting amphipods in rivers and streams might be exposed to UV radiation.

Little is known about the variation in pigmentation among acanthocephalan species, and even less about the potential role of carotenoid pigments in the protection of acanthocephalan cystacanths against UV radiation. According to the photoprotection hypothesis, the carotenoid content of cystacanths should be related to the risk of exposure to UV radiation (UVR) at the interspecific level. Thus, an important step to validate the adaptive function of cystacanth colouration in relation to UV protection is to experimentally investigate the effects of UVR on the survival of acanthocephalan cystacanths with different carotenoid content (see Cywinska et al., 2000; Armstrong et al., 2002; Lamare and Hoffman, 2004) and differential risk of exposure to UVR (Hansson and Hylander, 2009).

Here, we present new data on the carotenoid content and resistance to UVB in cystacanths belonging to three pigmented acanthocephalan species that all infest the amphipod *Gammarus pulex* (Fig. 1), but bring about contrasting behavioural alterations in their common intermediate host (Cézilly et al., 2000; Kaldonski et al., 2007; Perrot-Minnot et al., 2007). The two pomphorhynchid parasites *Pomphorhynchus laevis* and *Pomphorhynchus tereticollis* reverse the phototaxis of their intermediate host *G. pulex*, making infected amphipods increasingly attracted to light (Cézilly et al., 2000; Tain et al., 2006), while remaining benthic when uninfected (Perrot-Minnot, unpublished data) or, at least, slightly less benthic (*P. laevis*; Cézilly et al., 2000). In constrast, the bird polymorphid parasite *P. minutus* reverses geotaxis in its intermedidate host, resulting in infected *G. pulex* spending more time at the water surface (Cézilly et al., 2000; Perrot-Minnot, unpublished data). The negative phototaxis of *G. pulex* is not reversed by *P. minutus* (Tain et al., 2006; Perrot-Minnot, unpublished data) or only moderately so (Cézilly et al., 2000). Because exposure to UV is maximal at the water surface (Gáspár et al., 1996), *P. minutus* are presumably exposed to more UVR than the two fish acanthocephalans. We assessed the carotenoid content of cystacanths using HPLC analysis, and investigated the effects of UVB radiation in vitro on both the survival and genomic integrity of cystacanths. We discuss our results in relation to the role of carotenoids as photoprotective compounds, and to the evolutionary significance of their accumulation in larvae of acanthocephalan parasites.

2. Materials and methods

2.1. Collection of samples

Parasite samples were collected from three different rivers (Bèze, Ouche, Tille) in Burgundy (eastern France). Parasites were sampled by collecting infected *G. pulex* which were easily recognised in the field by the presence of a yellow-orange dot that corresponds to the cystacanth, visible through the translucid cuticle of the host. Parasite species were identified based on morphological characteristics and prior genetic analyses of the sampled populations (see Perrot-Minnot, 2004). For HPLC analysis of carotenoid content, cystacanths were rinsed in deionized water after dissection in saline, and quickly dried on absorbant paper. Between 16 and 25 conspecific individuals were then pooled together in a tube and stored at −80 °C. The exposure of live cystacanths to UVB radiation in vitro was performed directly after dissection in saline.

2.2. Extraction of carotenoids and HPLC analysis

Extraction of carotenoids was achieved following the protocol of Gaillard et al. (2004), using Matrix Solid Phase Dispersion (MSPD). Briefly, frozen parasites were crushed on dry ice and mixed with 200 mg of Isolute MSPD grade C18 sorbent material (International Sorbent Technology Ltd., UK). One-hundred microlitres of internal standard (2 ng/μL of lycopene in 50% methyl-*tert*-butyl ether (MTBE)/50% methanol) were applied on the column, before the pigments were eluted with 500 μL of methanol first and then 500 μL of MTBE. By using a combination of polar and non-polar solvents in the MSPD procedure, the complete extraction of carotenoids including xanthophylls and carotenes was achieved (Gaillard et al., 2004). After evaporation under nitrogen in the dark, parasite extracts were re-dissolved in 50 μL of 50% MTBE/50% methanol and injected into the HPLC column.

HPLC separations were performed according to Gaillard et al. (2004) 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 Millenium[32] software. Separation of 50 μL carotenoid extracts was carried out using a mixture of MTBE, methanol and deionized water as the mobile phase, following these steps: 10 min with methanol/MTBE/water (81:15:4 v/v/v) followed by a 40 min linear mobile phase gradient from 81:15:4 to 10:90:0 methanol/MTBE/water v/v/v, and a 20 min gradient back to initial conditions. These conditions allowed a complete elution of pigments from xanthophylls to non-polar carotenoids in one step (Gaillard et al., 2004). Absorption spectra were collected from 250 to 600 nm with a

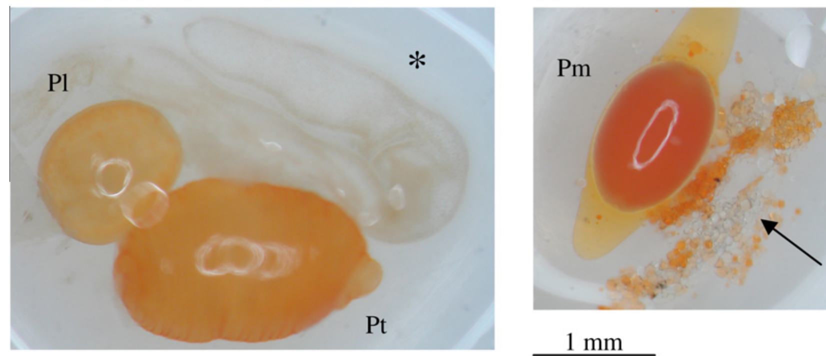

Fig. 1. Cystacanths of *Pomphorhynchus laevis* (Pl), *Pomphorhynchus tereticollis* (Pt) and *Polymorphus minutus* (Pm), dissected out from the hemocoele of their intermediate host, the amphipod *Gammarus pulex*. Asterisk: immature larvae (acanthella) not yet pigmented; arrow: pigmented lipid droplets from the amphipod's hemolymph.

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 (λ_{max} values) with pure standards and with carotenoids from *G. pulex* samples previously analysed by HPLC–MS coupling (Gaillard et al., 2004). The identification of carotenoids naturally present as esterified forms was improved through saponification conducted on dry parasite carotenoid extract with 500 μL of a methanolic 0.5% NaOH solution, prior to HPLC (see Gaillard et al., 2004). Calibration curves established from the regression of peak areas on known amounts of carotenoid standard were used to estimate the amount of astaxanthine, lutein and β-carotene in each parasite species. Carotenoid standards (astaxanthin, lutein, zeaxanthin, β-carotene and lycopene) were purchased from Extrasynthèse SA (Genay, France). The antioxidant 2,6-di-*tert*-butyl-*p*-cresol (BHT; Fluka Chemika, Switzerland) was added to HPLC-grade solvents (at 0.01%) to prevent degradation of carotenoids during extraction and separation.

Quantification of carotenoid content could not be confidently performed for all peaks since the resolution of some of them was not complete (especially for *P. laevis* samples), and because no carotenoid esters were available as commercial standards. A quantitative comparison of carotenoid content in the three parasite species was however performed through comparing the peak areas of five carotenoids or pooled carotenoids (astaxanthin, lutein, other xanthophylls and carotenes, esterified astaxanthin and other esterified compounds) after correcting for the number of cystacanths pooled in a sample (ranging from 16 to 25). An additional analysis was done after correcting for the mean volume of a cystacanth, thus standardising the amount of parasite tissue analysed in individual samples. Cystacanth volumes were estimated from previous data (not shown) for *P. laevis* (0.21 ± 0.065 mm^3, n = 1044), *P. tereticollis* (0.58 ± 0.19 mm^3, n = 117), *P. minutus* (0.19 ± 0.06 mm^3, n = 231).

2.3. UVB radiation

Since the main goal of our study was to establish a link between cystacanth pigmentation and tolerance to UVB radiation, a single acute exposure of cystacanths was done in vitro after being taken out of their amphipod hosts. Although such short-term high level exposure did not compare with the natural regime of exposure experienced by these temperate freshwater organisms, it avoided any residual variation due to uncontrolled factors such as movement, cuticle pigmentation or survival of the amphipod host. Three conspecific cystacanths were pooled in a volume of 100 μL of saline solution and exposed in the wells of a microtitre plate, at 15 °C under a UVB fluorescent lamp (Cole-Parmer, USA, VL6M 6 W, 312 nm). Since total energy received varied as a function of radient exposure intensity and exposure time, both parameters were monitored with a radiometer and its sensor placed closed to the samples (VLX-3W, Cole-Parmer, USA). To ensure the stability of emitted radiation at an intensity of 1.8 W cm^{-2}, the UVB-lamp was warmed for 1 h prior to each experiment. Controls were performed by placing cystacanths under the same conditions as irradiated ones in the experimental design, except for the addition of a plexiglass screen blocking UVB radiation.

Exposure duration ranged from 1 to 5 h for evagination tests (determined from preliminary tests). A lower range of exposure time was chosen to assess DNA damage, since altered evagination capacity possibly means a sublethal state and, thereby, a level of DNA degradation that could interfere with the quantification of cyclobutane pyrimidine dimers (CPDs, see below). The highest exposure duration to assess DNA damage was therefore chosen so as to allow at least 90% evagination. Total energy received was strongly and linearly correlated with exposure time in both experiments (Linear regression of total energy received in kJ m^{-2} on duration in minutes: Evagination experiment, R^2 = 0.98, $F_{1,50}$ = 2488, P < 0.0001; DNA damage experiment, R^2 = 0.997, $F_{1,46}$ = 19086.5, P < 0.0001). The average total energy received ranged from 0.58 kJ m^{-2} (1 h) to 2.76 kJ m^{-2} (5 h) for evagination tests, and from 0.06 kJ m^{-2} (5 min) to 1.33 kJ m^{-2} (2 h) for DNA damage assays. Although radiation intensity might be more than 500 times higher than natural UVB radiations, the total energy received under our experimental conditions would have corresponded to 1.9–72.5 days of natural underwater exposure for the evagination test, and from 4.3 h to 35 days for the DNA damage assay, based on daily UVB radiation registered in temperate coastal waters (from 3.81 to 31.2 kJ m^{-2}; Sugawara et al., 2003). The exposure doses used here are in the range of the total UVB energy received after 5–7 days exposure at 20 cm underwater at a subtropical site (100–250 kJ cm^{-2}; Schouten et al., 2008). After exposure, all samples were immediately processed (see Sections 2.4 and 2.5) to avoid repair processes.

2.4. Evagination test

The infectivity of cystacanths was assessed by their readiness to evaginate their proboscis when placed under environmental conditions close to those encountered in the lumen of their final host's digestive tract. This test is a surrogate for lethality after UVB exposure since evagination is a physiologically demanding and active process, allowing the parasite to establish in its final host (Hammond, 1966; Taraschewski, 2000). The two fish parasites, *P. laevis* and *P. tereticollis*, were placed in a solution of bile from eels at 15–17 °C in the dark. Bile from eels was reconstituted from lyophilised bile (Sigma, Ref. B 6518) resuspended at 0.2% in fish saline. The waterfowl parasite *P. minutus* was placed in distilled

water at 42 °C in the dark. Under these conditions, evagination usually occured within an hour and was easily recorded by visual inspection under a binocular microscope.

2.5. Quantification of radiation-induced DNA damage using ELISA

The genotoxic properties of UVB radiation (290–320 nm) mostly involve bipyrimidine DNA photoproducts (Cadet et al., 2005). The production of CPDs, typical of UVB-induced DNA damage, was quantified using a monoclonal primary antibody (TDM-2) (Mori et al., 1991; Armstrong et al., 2002). Genomic DNA was extracted from samples consisting of three pooled cystacanths using a standard phenol–chloroform protocol after incubation in Cetyldimethylethyl-ammonium bromide (CTAB) and RNAse treatment (Perrot-Minnot, 2004). Conspecific parasites were pooled three by three (as they were for UV exposure) to get enough DNA to run three replicates per plate. DNA pellets were resuspended in PBS (10 mM, pH 7.4). DNA concentration and quality were determined spectrophotometrically using 260:280 nm ratios (Smartspec, Bio-Rad, USA).

Fifty nanograms of DNA from each sample was used in ELISAs, and each sample was deposited randomly in triplicate in a polyvinylchloride flat-bottomed microtitre plate (Nunc, Maxisorb Immunoplate). We followed the procedure of Armstrong et al. (2002), except that blocking of non-specific sites was done with gelatine (1% in PBS). Primary antibody TDM-2 was used at 1:1000 and the secondary antibody, goat-anti-mouse IgG (H + L) conjugated with Biotin-F(ab′)$_2$ fragment (Zymed), was used at 1:4000. The absorbance was read at 490 nm with a microplate reader (Versamax, Molecular Devices) and analysed using the software SOFT-Max® Pro. 4.0 (Molecular Devices).

Three categories of controls were run in each plate: non-irradiated parasites (see Section 2.3), and DNA from calf thymus exposed or unexposed to UVB radiation. Aliquots of DNA from calf thymus (Sigma) resuspended in PBS at 50 ng/μL were directly exposed to a range of UVB radiation (0, 30, 60 and 180 s, at 1.8 W cm^{-2}). This range was chosen to give a linear response curve and a maximum of CPDs higher than that observed in parasite samples during preliminary tests. Non-irradiated parasites and DNA from calf thymus irradiated for 180 s at 1.8 W cm^{-2} were used as negative and positive controls, respectively, to correct for plate-to-plate variability. Following Armstrong et al. (2002), the corrected absorbance was calculated as: (Absorbance [UVB treated sample] – Absorbance [UVB negative control])/Absorbance [UVB positive control].

2.6. Statistical analyses

The analysis of carotenoid content from HPLC profiles was done on peak areas, after correcting for the number of pooled cystacanths per sample. For each of five carotenoids or pooled carotenoids (astaxanthin, lutein, other xanthophylls and carotenes, esterified astaxanthin and other esterified compounds), peak areas were compared between the three species, using a Kruskall–Wallis test, followed by post hoc paired comparisons (Siegel and Castellan, 1988). Šidák correction was used to correct for multiple tests.

Parasite species (categorical variable) and duration of exposure to UVR (continuous variable) were used as predictor variables of evagination rate in a logistic regression model. The nominal dependent variable was set as either 1 or 0, depending on whether or not the cystacanth evaginated its proboscis. Individual effects in the model were tested by Wald Chi square statistics.

CPD production, quantified by ELISA, was analysed using an analysis of co-variance (ANCOVA), with parasite species and exposure duration as predictor variables of corrected absorbance. All tests were performed using JMP statistical software (v. 5.0, SAS Institute Inc., USA).

3. Results

3.1. Carotenoid contents of P. laevis, P. tereticollis, and P. minutus

Fifteen samples were analysed, each including 16 to 25 individual cystacanths collected from the same population: six *P. minutus* samples (Bèze river), five *P. laevis* samples (Ouche river), and five *P. tereticollis* samples (Bèze river). Based on the retrieval rate of the internal standard (lycopene), we estimated the mean extraction and injection yield at 73.8% (SD = 0.1%), 66.9% (SD = 0.16%) and 80.2% (SD = 0.15%) for *P. laevis, P. tereticollis* and *P. minutus* samples, respectively. These values were used to correct for differences in extraction and injection yield in the calculation of peak areas for each carotenoid fraction prior to the analysis of carotenoid profiles.

HPLC traces of *P. laevis* appeared to contain xanthophyll (mainly astaxanthin and lutein), esterified astaxanthin and other esterified compounds (possibly esterified lutein) (Fig. 2). However, the low amount of carotenoid compounds made their identification difficult, especially for esterified forms. Saponification of four extracts of *P. laevis* mainly released lutein, with astaxanthin additionally found in two of them. In chromatograms of *P. tereticollis* extracts, six well separated peaks were identified as xanthophyll compounds, and several others as non-polar compounds (Fig. 2). The comparison of retention times and absorption spectra with authentic carotenoids and with carotenoids identified by mass spectrometry in *G. pulex* (Gaillard et al., 2004) allowed the identification of most of these carotenoids. Free astaxanthin, β-carotene, lutein, esterified astaxanthin and other esterified compounds, and several other xanthophylls also found in *G. pulex* (mainly zeaxanthin, β-cryptoxanthin, ββ-carotene-3,4,3′-triol, and 5,3-hydroxyechinenone) were found (Fig. 2). Four additional carotenoid samples of *P. tereticollis* were extracted using only MTBE, in order to extract only non-polar compounds, before saponification. All yielded mainly lutein, and in one of the four samples, some astaxanthin. Finally, HPLC traces of *P. minutus* samples confirmed our previous analyses (Gaillard et al., 2004), showing the presence of predominantly esterified astaxanthin in *P. minutus*. This was confirmed through saponification of carotenoid extracts from two additional samples prior to HPLC. A set of fractions with a lower retention time was unambiguously identified as esterified astaxanthin, possibly monoesters. These esterified forms of astaxanthin amounted to 27% (SD = 3%) of total esterified astaxanthin in *P. minutus*, but were not found in samples of *P. laevis* nor in *P. tereticollis.*

The quantitative comparison of carotenoid content between the three parasite species (Table 1; Fig. 2A) revealed significant differences in peak areas for free astaxanthin (Kruskall–Wallis test: $\chi^2 = 9.97$, d.f. = 2, $P < 0.05$), lutein ($\chi^2 = 10.74$, d.f. = 2, $P < 0.05$), other xanthophylls and carotenes ($\chi^2 = 10.44$, d.f. = 2, $P < 0.05$) and esterified astaxanthin ($\chi^2 = 13.34$ d.f. = 2, $P < 0.01$), but not for pooled other esterified compounds ($\chi^2 = 8.5$, d.f. = 2, $P > 0.05$). After correcting for cystacanth volume (Table 1; Fig. 2B), significant differences were found in peak areas for free astaxanthin ($\chi^2 = 9.78$, d.f. = 2, $P < 0.05$), lutein ($\chi^2 = 10.17$ d.f. = 2, $P < 0.05$), other xanthophylls and carotenes ($\chi^2 = 10.44$, d.f. = 2, $P < 0.05$), esterified astaxanthin ($\chi^2 = 11.86$ d.f. = 2, $P < 0.05$), and other esterified compounds ($\chi^2 = 10.8$, d.f. = 2, $P < 0.05$). In post hoc paired comparisons, *P. laevis* always had a significantly lower amount of carotenoids compared with either of the two other parasite species (Table 1; Fig. 2A and B).

3.2. Capacity of cystacanths to evaginate their proboscis after UV exposure

The proportion of controls (cystacanths exposed to UVB radiation through a plexiglass screen) able to evaginate their proboscis

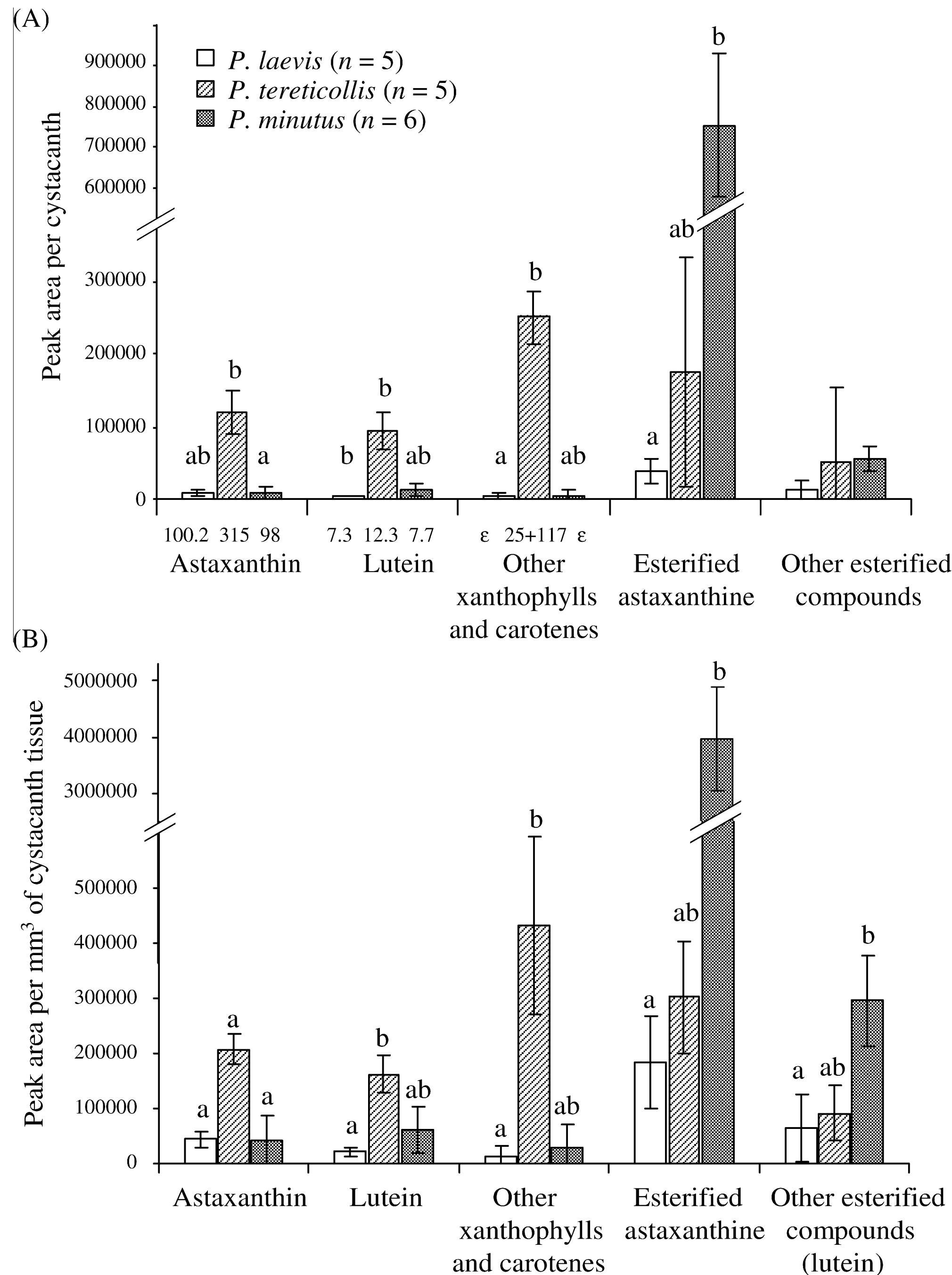

Fig. 2. Carotenoid composition in cystacanths of three acanthocephalan parasite species (*Pomphorhynchus laevis*, *Pomphorhynchus tereticollis* and *Polymorphus minutus*), given as peak areas from HPLC analysis (mean ± SD) per cystacanth (a) and per mm^3 of cystacanth tissue (b). The analysis was conducted on 16–25 pooled individuals. Bars not connected by the same letter are significantly different (Kruskall–Wallis test and post hoc paired comparison, $P < 0.05$ after Šidák correction for multiple testing). Numbers below bars correspond to the amount of carotenoid in ng per cystacanth (A) or per mm^3 of cystacanth tissue (B) estimated from calibration curves. For other xanthophylls and carotenes, this quantification could be done only for zeaxanthin + β-carotene, in *P. tereticollis*.

was equal or close to 100% (Fig. 3), and did not differ between parasite species nor according to the duration of exposure (logistic regression, $\chi^2 = 4.51$, d.f. = 5, $P = 0.48$). These data were therefore pooled for further analysis and are thereafter referred to as '0 h'.

Following exposure to UVB, the ability of cystacanths to evaginate their proboscis differed significantly between parasite species and decreased significantly with exposure duration from 0–4 h (logistic regression: $N = 829$; $\chi^2 = 199$, d.f. = 5, $P < 0.0001$; parasite species: $\chi^2 = 117.3$, d.f. = 2, $P < 0.0001$; exposure duration: $\chi^2 = 60.4$, d.f. = 1, $P < 0.0001$) (Fig. 3). There was a significant interaction between parasite species and exposure duration ($\chi^2 = 12.7$, d.f. = 2, $P = 0.002$) (Fig. 3). Paired comparisons showed that the ability to evaginate was significantly lower in *P. laevis* compared with both *P. tereticollis* (logistic regression: $n = 576$; $\chi^2 = 122.3$, d.f. = 3, $P < 0.0001$; parasite species: $\chi^2 = 65.6$, d.f. = 1, $P < 0.0001$; exposure duration: $\chi^2 = 28.5$, d.f. = 1, $P < 0.0001$) and *P. minutus* (logistic regression: $n = 619$; $\chi^2 = 130.04$, d.f. = 3, $P < 0.0001$; parasite species: $\chi^2 = 62.9$, d.f. = 1, $P < 0.0001$; exposure duration: $\chi^2 = 46.8$, d.f. = 1, $P < 0.0001$) (Fig. 3). The interaction of parasite species and exposure duration was significant in the latter ($\chi^2 = 11.17$, d.f. = 1, $P < 0.0001$) but not the former ($\chi^2 = 3.58$, d.f. = 1, $P = 0.06$). There was no significant difference in the ability to evaginate, after UV exposure from 0–5 h, between *P. tereticollis* and *P. minutus* (logistic regression: $n = 670$; $\chi^2 = 118.9$, d.f. = 3,

Table 1
Differences in carotenoid content between cystacanths of three acanthocephalan species, *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis* and *Polymorphus minutus*, in ng per cystacanth (A) or in ng per mm^3 of cystacanth (B). Quantification was done only for carotenoids for which standards were used and peaks were resolved. The samples analysed by HPLC with electrochemical detection (HPLC-ED) (*n*) consisted of 16–25 snap-frozen pooled cystacanths (see text for detailed methodology).

Parasite species	*n*	Astaxanthin	Lutein	Zeaxanthin	Carotene
Per cystacanth					
P. laevis	5	18.45 ± 5.7	0.28 ± 0.11	1.91 ± 4.27	5.15 ± 11.52
P. tereticollis	5	238.76 ± 31.59	5.75 ± 1.23	99.87 ± 11.12	139.22 ± 22.59
P. minutus	6	16.15 ±16.68	0.71 ± 0.48	8.65 ± 11.29	0.00
Per mm3 of cystacanth					
P. laevis	5	87.87 ± 27.17	1.33 ± 0.52	9.09 ± 20.33	24.53 ± 54.84
P. tereticollis	5	411.66 ± 54.46	9.92 ± 2.13	172.19 ± 19.18	240.03 ± 38.95
P. minutus	6	84.97 ± 87.80	3.75 ± 2.55	45.51 ± 59.41	0.00

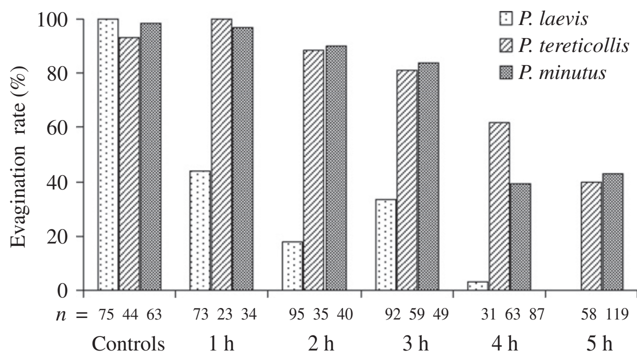

Fig. 3. Sublethality of UVB radiation in cystacanths of three species of pigmented acanthocephalan parasites (*Pomphorhynchus laevis, Pomphorhynchus tereticollis* and *Polymorphus minutus*) exposed to increasing doses in vitro. Sublethality was assessed from the ability of cystacanths to evaginate their proboscis following exposure. Controls: cystacanths exposed under UVB-blocking plexiglass screen (see Section 2.3). Numbers refer to sample sizes.

$P < 0.0001$: parasite effect, $\chi^2 = 0.98$, $P = 0.32$; exposure duration, $\chi^2 = 81.56$, $P < 0.0001$; interaction, $\chi^2 = 0.8$, $P = 0.07$; Fig. 3).

3.3. DNA damage: CPD formation

Because sublethal doses of radiation could affect DNA integrity, we assessed the quantity of CPDs produced after exposure to doses of UVB radiation allowing at least 90% evagination. Maximum exposure duration was thus 2 h for *P. tereticollis* and *P. minutus* (Fig. 3). Because the rate of evagination of *P. laevis* dropped drastically between 30 min (92%, $n = 48$) and 1 h (44%, $n = 73$) (Fig. 3 and data not shown), maximum exposure duration for this species was set at 30 min.

UV-induced DNA damage was quantified by the corrected absorbance results from a standard ELISA test, using primary antibody anti-CPDs. There was no difference in the amount of DNA damage between parasite species in samples exposed to UVB under UV-blocking plexiglass screen (controls 0 min, Fig. 4; ANOVA, $F_{3,32} = 0.35$; $P = 0.78$). The amount of DNA damage was compared between the three parasite species for durations of exposure ranging from 5–30 min. Both parasite species and duration of exposure contributed significantly to the observed variation in CPD formation (ANCOVA: $F_{5,\ 98} = 12.88$, $P < 0.0001$: parasite effect $F_{2,5} = 12.69$, $P < 0.0001$; duration effect $F_{1,5} = 40.8$, $P < 0.0001$), with no significant effect of the interaction between the two variables ($F_{2,5} = 2.11$, $P = 0.13$). CPD formation in *P. laevis* was consistently higher than in *P. tereticollis* and *P. minutus* (Fig. 4). CPD formation was then compared between *P. tereticollis* and *P. minutus* for longer exposure durations, up to 120 min. No significant effect of parasite species was evident (Fig. 4; ANCOVA: $F_{3,102} = 102$, $P < 0.0001$; parasite effect $F_{1,3} = 3.71$, $P = 0.06$; exposure duration effect $F_{1,3} = 90.9$, $P < 0.0001$; interaction $F_{1,\ 3} = 0.36$, $P = 0.55$).

4. Discussion

UV radiation, particularly UVB, is known to cause severe damage to aquatic organisms (Cywinska et al., 2000; Armstrong et al., 2002; Sinha and Häder, 2002; De Lange and Van Reeuwijk, 2003; Lesser and Barry, 2003; Häder et al., 2007; Hansson and Hylander, 2009; Krapp et al., 2009; Nazari et al., 2010). Carotenoids have well known antioxidant properties (Lesser, 2006), in particular as scavengers of photoproduced ROS (Bast et al., 1998; Young and Lowe, 2001; Stahl and Sies, 2003; Lamare and Hoffman, 2004; Hansson and Hylander, 2009). They may also act, to a certain extent, as screening compounds against UVR (Cockell and Knowland, 1999; Lamare and Hoffman, 2004). However, the ability of carotenoids to provide protection against UVR differs between compounds (Britton, 1995).

Our results show that observable differences in colour between cystacanths of different acanthocephalan species (Fig. 1; Kaldonski et al., 2009) correspond to qualitative and quantitative differences in carotenoid content, with potential differences in photoprotective properties. Whereas previous studies reported a relatively simple carotenoid content for acanthocephalan parasites (Barrett and Butterworth, 1973; Taraschewski, 2000; Duclos, 1996. Functional significance of pigment in larval *Corynosoma constrictum* Van Cleave, 1918 (Acanthocephala: Polymorphidae). PhD dissertation, University of Nebraska, USA), the present study provides a more complex view of the variation in carotenoid content. In particular, we believe that we show for the first time that several carotenoid pigments and their esters can co-exist within the cystacanths of some species. Such diversity is particularly high in *P. tereticollis,* where at least eight different compounds co-exist. This progress was achieved through the use of recent, more refined techniques of extraction and separation (Gaillard et al., 2004), particularly suitable for the analysis of biological samples rich in lipids and resistant to disruption, such as acanthocephalan cystacanths. The predominance of large amounts of esterified astaxanthin in cystacanths of *P. minutus* was confirmed, as previously identified by Barrett and Butterworth (1968) and Gaillard et al. (2004). Astaxanthin was also present both in free and esterified forms in *P. laevis* and *P. tereticollis*. These results reflect the predominance of this xanthophyll in their common intermediate host *G. pulex*, where it makes up 40% of total carotenoid content in weight (Gaillard et al., 2004). Significant quantitative differences in carotenoid content were found, however, between *P. laevis* and the other two species, as well as qualitative differences between *P. tereticollis* and *P. minutus*, in the relative importance of free and esterified forms. The observed interspecific variation in carotenoid content may simply reflect differences in the availability of carotenoids inside the intermediate hosts. However, cystacanths of the three species were obtained from the same intermediate host species, and the carotenoid content of *G. pulex* did not differ between populations (M.J. Perrot-Minnot, M. Gaillard and F. Cézilly, unpublished

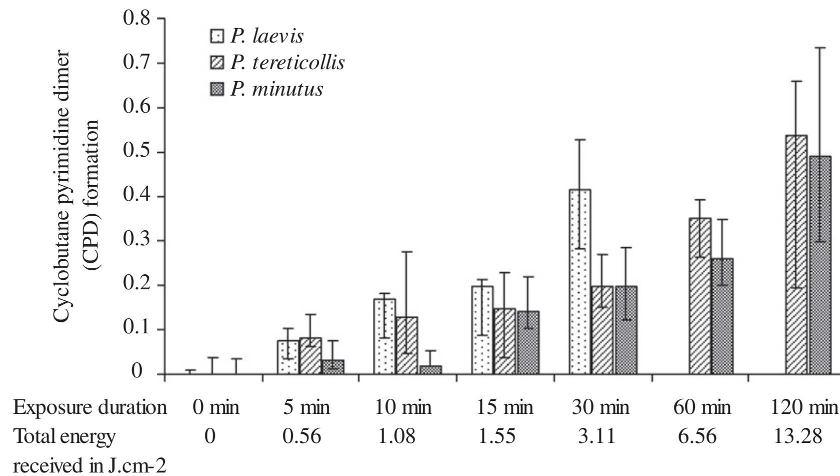

Fig. 4. Damage to DNA (median and quartiles) in three species of pigmented acanthocephalan (*Pomphorhynchus laevis, Pomphorhynchus tereticollis* and *Polymorphus minutus*) following in vitro exposure to increasing doses of UVB (given as exposure duration in min at 1.8 W cm^{-2} and as total energy received). Damage to DNA was measured as the amount of cyclobutane pyrimidine dimer (CPD) photoproducts in 50 ng of DNA extracted from exposed cystacanths (pooled by 3 ± 1). CPD formation was measured using an ELISA specific for this form of DNA damage, and is given as the absorbance corrected for minimum and maximum damage to control DNA (see Section 2.5).

results). Therefore, our results indicate that the three acanthocephalan species differ in their ability to selectively absorb carotenoids from their host and/or in their ability to metabolize carotenoids following absorption.

The in vitro acute exposure of cystacanths to high UVB radiation induced deleterious effects, both on the ability to evaginate and on DNA integrity. Although the exposure regime chosen oversimplified the natural UV environment in several ways and overestimated the actual UVB radiation experienced by these parasites, it allowed us to reveal interspecific differences in sensitivity to UVB, and to address its co-variation with carotenoid content. In addition, the total energy received corresponds to natural doses of UVB measured underwater after a few days to 2 months exposure in other ecosystems (Sugawara et al., 2003; Schouten et al., 2008). Exposure to UVB radiation revealed a higher susceptibility of *P. laevis* cystacanths compared with *P. tereticollis* and *P. minutus*, both in terms of sublethality (decreased evagination rate) and damage to DNA (increased cyclobutane pyrimidine dimers production). No difference in susceptibility to UVB radiation was evidenced between *P. tereticollis* and *P. minutus,* both species showing comparable levels of evagination rate and DNA damage. The deleterious effects of UVB reported here contrast with the study of Duclos (Duclos, 1996. Functional significance of pigment in larval *Corynosoma constrictum* Van Cleave, 1918 (Acanthocephala: Polymorphidae). Ph.D. Dissertation, University of Nebraska, USA) on two bird acanthocephalan species. Duclos (Duclos, 1996. Functional significance of pigment in larval *Corynosoma constrictum* Van Cleave, 1918 (Acanthocephala: Polymorphidae). Ph.D. Dissertation, University of Nebraska, USA) found no effect of chronic exposure to natural doses of UVB radiation on the development and viability of *Corynosoma constrictum* and *Polymorphus trochus* larvae in their amphipod host *Hyalella azteca.* Hence, and despite differential accumulation of carotenoid pigments between these two polymorphid species, the photoprotective role of carotenoids could not be addressed. Although the exposure regime used in this study is probably closer to those experienced naturally (long term in vivo exposure), a comparison with our data is limited by the fact that it did not mention the intensity of UVB nor the total energy received.

Overall, our results are consistent with a protective role of carotenoids against UVB radiation, since the species with the lowest amount of carotenoid (*P. laevis*) was the most susceptible. This is in agreement with numerous studies reporting on the photoprotective properties of carotenoids (Hansson and Hylander, 2009). However, the fact that *P. minutus*, the species with the richest content in astaxanthin, did not show greater protection against UVB than a species with a more diversified spectrum of carotenoid compounds, *P. tereticollis*, suggests that this particular carotenoid does not provide better protection than others, despite its supposedly high antioxidant activity. Indeed, although astaxanthin has been reported to show higher quenching capacity of singlet oxygen and scavenging capacity of ROS compared with other related carotenoids (Miki, 1991; Lawlor and O'Brien, 1995; Naguib, 2000), the relative efficiency of different carotenoids both as screening compounds and as antioxidants is still debated (Cockell and Knowland, 1999; Young and Lowe, 2001). In the present study, the photoprotective role of carotenoids against UVB radiation could only have come from their antioxidant properties, since carotenoids absorb UVA wavelengths, but very little UVB. A complementary role of carotenoids as screening compounds against UVA radiation should therefore be investigated in these acanthocephalan larvae, to fully understand the adaptive significance of interspecific differences in carotenoid content in relation to UVR.

Based on the hypothesis that the carotenoid-based colourations of acanthocephalans evolved as a protective device against UVR passing through the translucid cuticle of their crustacean hosts, it was predicted that the tolerance to UVR would increase with increased exposure rate under natural conditions, in relation to the behavioural alterations induced by each parasite in their common intermediate host. However, despite contrasting patterns of behavioural manipulation between *P. tereticollis* and *P. minutus*, suggestive of a higher exposure to UVB in the latter, and despite distinct carotenoid contents, both species were equally resistant to UVB. In addition, the positive phototactism induced by the two fish acanthocephalan species, *P. laevis* and *P. tereticollis*, did not imply similar levels of tolerance to UVB radiation nor of carotenoid content. Therefore, the evolution of carotenoid-based colouration in acanthocephalans appears to be unrelated to the consequences of behavioural alterations induced by the parasites. One possibility is that other photoprotective strategies covary with UVR experienced by manipulated infected gammarids in their microhabitat. Indeed, carotenoid accumulation is only one of the mechanisms by which plants and animals protect themselves from UV radiation or repair consequential damage. Other ubiquitous compounds such as mycosporine-like amino acids (MAAs) may play an important role as UV-absorbing compounds, as reported

in particular in aquatic invertebrates (Hansson and Hylander, 2009). If MAAs are present in the hemolymph of *G. pulex*, then the acanthocephalan larvae will be protected from UV radiation without requiring its own photoprotective compounds. In addition, the differential uptake of MAAs by acanthocephalan larvae according to UVR exposure risk should be addressed, as we did here for carotenoids.

An alternative hypothesis for the evolution of carotenoid accumulation by acanthocephalan parasites during larval growth relies on their physiology. Indeed, as lipophilic molecules, carotenoids tend to accumulate in membranes and other lipophilic compartments and to associate with lipoproteins (Stahl and Sies, 2003). Acanthocephalans store large amounts of lipids from their crustacean hosts, making up more than 25% of the dry weight of *P. minutus* cystacanths for instance (Taraschewski, 2000). These lipids are essentially stored in the external layers of the cystacanth body, partly as long-chain fatty acids (Crompton, 1970), where the carotenoids accumulate. In their intermediate host, carotenoids circulate in the hemolymph, bound to lipoprotein complexes or enclosed in lipid droplets (Barrett and Butterworth, 1968; Łotocka and Styczyńska-Jurewicz, 2001) (Fig. 1). One possibility is that the differential uptake of carotenoids between acanthocephalan species is simply a by-product of lipid uptake. Such a selective uptake has been hypothethized to account for variation in the accumulation of carotenoids between acanthocephalan species (Barrett and Butterworth, 1968) or between acanthocephalan parasites and their hosts (Gaillard et al., 2004). Alternatively, parasite species may differ in the way they metabolize carotenoids or store the precursor pigments of astaxanthin. On average, astaxanthin accounted for 40% of total carotenoid content, by weight, of uninfected female *G. pulex*, while lutein (20%) and ββ-carotene-3,4,3′-triol (possibly an intermediate form in the biosynthetic pathway from β-carotene to astaxanthin) were the others major pigments (Gaillard et al., 2004). *Pomphorhynchus laevis* would passively uptake lutein predominantly, and astaxanthin, with barely any conversion, whereas *P. minutus* would completely convert carotenoids into astaxanthin, followed by esterification in the fatty-acid rich layer. *Pomphorhynchus tereticollis* would store both lutein and astaxanthin, but also a range of intermediate xanthophylls in the biosynthetic pathway from possibly β-carotene to astaxanthin (see Berticat et al., 2000 for a putative biosynthetic pathway). Since lipids and proteins are sensitive to oxidative stress (Lesser, 2006), carotenoid uptake could be part of the antioxidant defenses of gammarids, but also of that of parasites. The role of carotenoids as antioxidant compounds should thus be addressed, through measuring several biomarkers of oxidative defenses or oxidative damage in acanthocephalan larvae differing in carotenoid uptake and in their crustacean hosts (Lesser, 2006; Krapp et al., 2009; Nazari et al., 2010).

In future studies, we will apply the same techniques used here to determine the carotenoid content of cystacanths in a larger range of acanthocephalan species. Extending such a comparative study of carotenoid content to diverse species throughout the entire order Palaeacanthocephala (in which pigmented species are distributed) will help to understand the evolution of carotenoid-based colouration in these parasites, provided some ecological and physiological traits are determined. Among ecological constraints and physiological attributes of white and pigmented parasites, UV threat levels (related to the pattern of behavioural modification induced in and the ecology of their intermediate host) and lipid and protein content should be documented. Comparative methods (Harvey and Pagel, 1991; Pagel, 1997), taking into account the available information on acanthocephalan phylogeny (García-Varela et al., 2002; Near, 2002; Herlyn et al., 2003), would then allow disentangling of the relative effect of phylogenetic constraints versus ecological or physiological traits on carotenoid accumulation. From a more ecological point of view, to fully apprehend the functional role of parasites in ecosystems, one should take into account not only the role of parasites in network topology (Ings et al., 2009), or in energy flow or biomass in food webs (Lafferty et al., 2008), but also their role in the transit of molecules of major biological function such as carotenoids.

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