Astaxanthin in the calanoid copepod *Calanus helgolandicus*: dynamics of esterification and vertical distribution in the German Bight, North Sea

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ABSTRACT: Zooplankton synthesise astaxanthin, a carotenoid pigment believed to protect against high-energy irradiance, from precursors in their diet. Different patterns of astaxanthin vertical distribution would be expected from the benefits of photoprotection, the costs of visual predation and the availability of food. Despite a highly resolved sampling approach (4 m depth intervals), no clear pattern of vertical distribution or correlation with chlorophyll *a* concentrations was found for *Calanus helgolandicus* astaxanthin concentrations in the German Bight. This may be attributable to photochromatic adaptation in phytoplankton, diurnal rhythms of copepod grazing and, particularly, vertical migration. A 2×2 factorial (light × food) experiment showed that total astaxanthin concentrations in *C. helgolandicus* were entirely determined by the dynamics of esterified astaxanthin. As expected, the concentrations of astaxanthin esters decreased in starved individuals in the dark, while the combined presence of food and light resulted in an increase of esterified astaxanthin. Similar levels of esterified astaxanthin were maintained in both starving individuals exposed to light and feeding individuals in the dark. The latter implies that astaxanthin esterification is *per se* a process independent of light, although light exposure enhances it. It is proposed that the function of astaxanthin esters is to generally improve the antioxidant protection of storage lipids, also in situations where photoprotection is not required.

KEY WORDS: Astaxanthin · Carotenoids · Pigments · Photoprotection · Vertical migration

INTRODUCTION

Zooplankton synthesise astaxanthin, a carotenoid pigment, from precursors in their diet (Goodwin 1984). Chemically, it is found in the free (unesterified) form, as mono- and di-esters of fatty acids or bound to proteins forming carotenoproteins. Because of its strong antioxidant ability, astaxanthin is believed to protect zooplankton from photo-oxidative damage caused by high-energy irradiance (Hairston 1978). This view is supported by experimental studies showing the higher survival of intensely pigmented compared to transparent individuals exposed to ultraviolet (UV) or blue light (Hairston 1978, Ringelberg et al. 1984, Davenport et al.

2004). Furthermore, zooplankton have been shown to respond differentially to UV exposure, with less-pigmented species descending farther from the light source than more intensely pigmented ones (Rhode et al. 2001).

Studies on the vertical distribution of carotenoids in freshwater zooplankton are generally consistent with a photoprotective function, i.e. the more pigmented individuals occur closer to the surface (Hairston 1980, Byron 1982, Hansson 2000). In contrast, the more pigmented zooplankton in the sea are often found to occur deeper (Herring 1972) and to avoid the sea surface (<6 m) during the daytime (Hays et al. 1994). Thus, while the latter, marine results are consistent with pre-

dictions of surface avoidance as a means of reducing the increased predation risk from visual predators (Hairston 1979, Luecke & O'Brien 1981, Byron 1982), they are clearly difficult to bring in line with a photoprotective function of astaxanthin.

Here, we report on the vertical distribution of astaxanthin concentrations in the copepod *Calanus helgolandicus* in the German Bight, North Sea, during summer. We adopted a high-resolution sampling approach with sampling at 4 m depth intervals, given that blue and UV light is generally completely attenuated within the top 2 to 10 m in temperate coastal waters (Jerlov 1976, Kuhn et al. 1999). We expected one of the following: (1) a decrease is astaxanthin with increasing depth due to photoprotection, (2) an increase with increasing depth due to visual predation, (3) a 'trade-off', with lowest concentrations at intermediate depths, or (4) a positive relationship with phytoplankton biomass, since astaxanthin synthesis depends on the ingestion of precursor carotenoids. In addition, we performed an 2×2 factorial onboard experiment to test for the effects of food and light on the temporal dynamics of astaxanthin.

MATERIALS AND METHODS

Zooplankton were collected at 3 stations in the German Bight, North Sea, from 9 to 11 August 2004. The stations were sampled in the order: Stn 11 (7° 40' E, 54° 10' N) at 15:00 h; Stn 22 (7° 00' E, 54° 20' N) at 23:00 h; and Stn 35 (5° 00' E, 54° 40' N), at 01:00 h and again at 13:00 h. Surface (<0.5 to 1 m) and subsurface (4 to 28 m) samples were collected with a Bongo net (310 µm mesh size) and a multiple opening–closing net (335 µm mesh size) at 4 m intervals, respectively. After sampling (~1.5 h) and gut evacuation (~1 h), zooplankton were collected on sieves (~1.5 h) and subsequently stored in liquid nitrogen. Thus, the total time elapsed between sampling initiation and fixation was ~4 h.

An onboard experiment was conducted with copepods collected on 28 July 2005 at the chl *a* maximum (~42 m) of a deep (60 m) station (3° 27' E, 56° 39' N). The experimental design was 2×2 factorial, with food or no food addition crossed with light or no light exposure (4 treatments $\times$ 2 replicates). Copepods were added to a total of 8 buckets (20 l) containing either GF/F-filtered seawater or unfiltered seawater from the chl *a* maximum; 2 buckets of each group were covered with black foil or exposed to continuous white light provided by 2$\times$ 58W/30 Osram[TM] fluorescent lamps. The light intensity (50 µmol m^{-2} s^{-1}) was adjusted to that of the chl *a* maximum at noon. Gentle aeration was provided to all buckets to prevent phytoplankton from sinking. Zooplankton samples were taken at 0, 4, 12

and 24 h after the onset of the experiment and immediately stored in liquid nitrogen.

In the laboratory, females and Stage 5 copepodites of *Calanus helgolandicus* were separated under a dissecting microscope and collected on small (~5 $\times$ 10 mm) pieces of GF/F filter. Generally, all available individuals of a sample (20 to 30) were pooled in order to obtain 1 measurement per depth interval. After selection, the copepodites were stored at –80°C for ~2 mo until pigment analysis by high-performance liquid chromatography (HPLC). Re-freezing the samples after selection had no significant impact on the astaxanthin concentrations (Student's *t*-test, n = 8, p > 0.4).

Pigments were extracted by placing the filters into 5 ml syringes capped with a 0.2 µm filter, together with 1000 µl acetone. For the onboard samples, 2 ml syringes and 500 µl acetone were used. After sonication (~10 s) and extraction in the dark (24 h at –20°C), the copepod and filter-remains were removed by squeezing the extract through the filter. Aliquots (100 µl) of the filtrate were measured in a Shimadzu HPLC-system equipped with an LC18 cartridge column (25 cm $\times$ 4.6 mm $\times$ 5 µm) and a photodiode-array detector. The solvent system (Solvent A: 80% methanol, 20% 0.5 M ammonium acetate; Solvent B: 90% acetonitril, 10% water; Solvent C: 100% ethylacetate) used the linear gradient 0 min: 100% A; 2 min: 100% B; 2.6 min: 85% B, 15% C; 15 min: 29% B, 71% C; 25 min: 100% B; 27 min: 100% A. Free astaxanthin and its esters (4 to 6 peaks) were identified by the retention time and/or absorption spectrum of commercially available astaxanthin (Sigma[TM]).

Copepod dry weight (DW) was estimated from length measurements using length: DW regressions (Mauchline 1998). Chl *a* was determined spectrophotometrically from samples (500 ml) extracted in 90% acetone (24 h).

Using the GLM procedure of SAS[TM], multivariate analysis was conducted with depth, station and time of sampling (day/night) as independent variables. Nonsignificant (p > 0.05) variables were removed in a backward, stepwise process. Regressions on timebased astaxanthin concentrations were performed using SigmaPlot[TM] 9.0 software.

RESULTS

Calanus helgolandicus was found at all depth intervals of stations sampled at night, but was absent from the surface and deeper depth intervals in the daytime, particularly at Stn 11 (Fig. 1). Overall, the concentrations of astaxanthin were low (<0.22 µg mg^{-1} DW) and seemed unrelated with depth. However, multivariate analysis revealed a significant correlation of the total
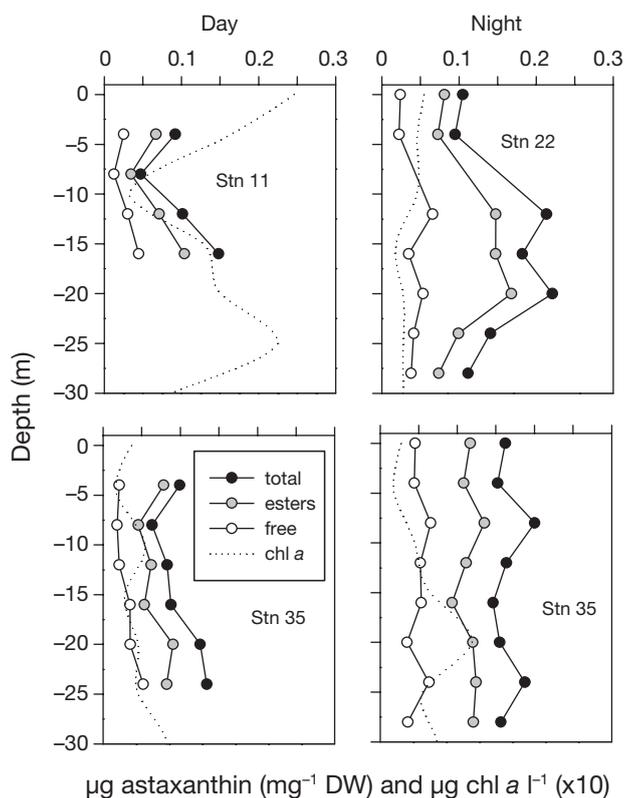
Fig. 1. *Calanus helgolandicus*. Chl *a* and copepod astaxanthin concentrations as a function of depth. No astaxanthin data are available for the 8 m depth interval at Stn 22
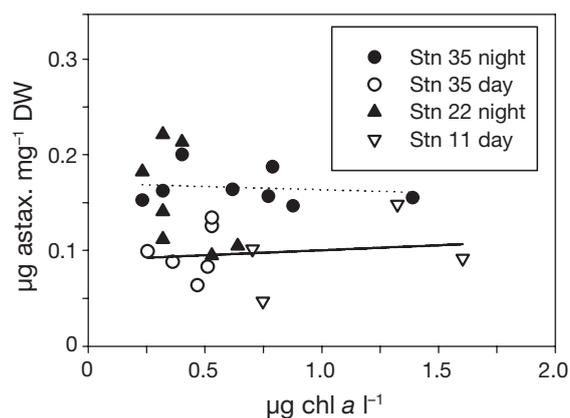

Fig. 2. *Calanus helgolandicus*. Total astaxanthin concentrations as a function of chl *a*. Dotted and continuous lines = least-square linear regressions ($r^2 < 0.05$; $p > 0.7$) for night and day, respectively

and esterified astaxanthin concentrations with depth (Table 1). This was due to the somewhat lower concentrations at the 4 and 8 m depth intervals compared to the preceding surface and 4 m depth intervals, respectively.

Similarly, the vertical distribution of astaxanthin seemed unrelated with the vertical distribution of chl *a*, with the possible exception of Stn 11 (Fig. 1). Plotting total astaxanthin as a function of chl *a* yielded no significant correlation (Fig. 2). Instead it showed that

the concentrations of total astaxanthin were on average 60% higher at night than during the day. Multivariate analysis revealed that the difference between night and day concentrations was highly significant for both total and esterified astaxanthin, yet insignificant for free astaxanthin (Table 1). In turn, free astaxanthin were found to vary significantly with station; this was due to the combination of relatively low and high concentrations at Stns 11 and 35, respectively.

At Stn 35 (the only station sampled both day and night), the strongest diurnal differences (~0.04 to 0.12 µg mg^{-1} DW) were found at the 8 m depth interval for all astaxanthin classes (Fig. 3). These differences correspond to increases of ~180 to 230% of the respective day concentrations. Overall, astaxanthin esters represented ~60 to 80% of the total astaxanthin concentration. At Stn 35, the degree of astaxanthin esterification was marginally higher during the day than at night within the upper ($\geq$12 m) water column, whereas the opposite was true for depths >12 m. Multivariate analysis detected no significant correlation of % astaxanthin esters with depth, time or station (Table 1).

Table 1. *Calanus helgolandicus*. Results of multivariate analysis of astaxanthin concentrations and percent esterified astaxanthin using depth, time of sampling (day/night) and station as independent variables. Data were square root transformed to reduce heteroscedasticity. Statistically significant (p < 0.05) results are in boldface

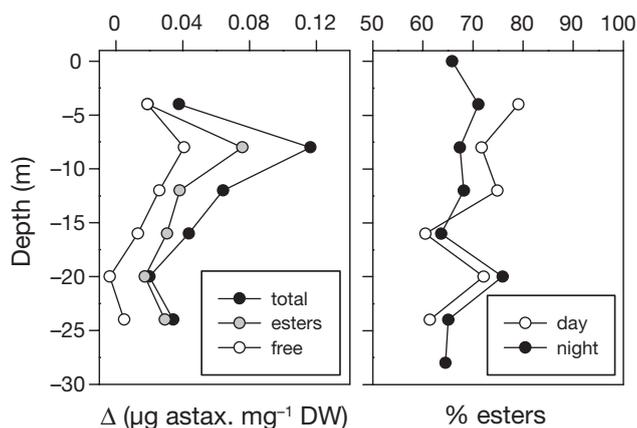| Independent variable	df	Dependent variable											
		Total astaxanthin			Esterified astaxanthin			Free astaxanthin			% esterified astaxanthin		
		MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
Depth	1	0.01097	6.66	**0.018**	0.00748	6.77	**0.017**	0.19754	1.25	>0.3	0.00049	0.08	>0.7
Time (day/night)	1	0.02155	13.08	**0.002**	0.03048	27.60	**<0.0001**	0.11269	0.72	>0.4	0.00075	0.12	>0.6
Station	2	0.00028	0.17	>0.8	0.00238	2.15	>0.2	0.72526	4.60	**0.023**	0.00053	0.09	>0.7

Fig. 3. *Calanus helgolandicus*. Diurnal (night/day) changes in astaxanthin concentrations and % esters at Stn 35. Astaxanthin data shown only for 4 to 24 m depth interval, since daytime data lacking for surface and 28 m intervals

The initial samples of the onboard experiment, which were fixed immediately after sampling, contained ~20 to 70% higher astaxanthin concentrations (0.12 to 0.29 µg mg^{-1} DW, Fig. 4) than the field samples set aside for gut evacuation experiments. In treatments without food (Fig. 4: 'No food'), the concentrations of total astaxanthin decreased exponentially over time, both in the dark and (less pronounced) in the light. The concentrations of free astaxanthin decreased in both treatments by ~40 to 50% after 4 and 24 h, respectively. In turn, the concentrations of esterified astaxanthin were sustained under light exposure, but decreased exponentially in the dark. Consequently, the degree of astaxanthin esterification at the end of the experiment was higher in starved individuals exposed to light (~73%) than those kept in the dark (~67%).

In treatments with water from the chl *a* maximum (Fig. 4: 'Food'), total astaxanthin concentrations decreased in the dark, but remained at approximately constant levels in the light. Free astaxanthin concentrations decreased exponentially over time, irrespective of the presence or absence of light, and thus resembled the pattern observed in the 'no food' treatments. In contrast, astaxanthin esters increased over time in individuals exposed to both food and light, while they were sustained at approximately initial levels in feeding individuals in the dark. The sustained levels of total astaxanthin in the light × food treatment were the result of a quantitatively similar (~0.06 µg mg^{-1} DW) increase and decline in the esterified and free astaxanthin fraction, respectively. At the end of the experiment, the degree of esterification was on average slightly higher in feeding individuals exposed to light (~78%) than in those in the dark (~75%), although in both cases it was higher than in starved individuals (~67 to 73%).

DISCUSSION

The most striking result of this study is that our sampling approach provided no conclusive picture of the factors governing the vertical distribution of astaxanthin in *Calanus helgolandicus*. This finding is in contrast to both our experimental results and previous field studies (Hairston 1980, Byron 1982, Hays et al. 1994, Hansson 2000), which demonstrated the important role of food and light on carotenoid pigmentation in zooplankton. Several reasons may account for this fact.

First, the use of chl *a* as an estimate of the potential ingestion of dietary carotenoids may be inadequate, since the composition of phytoplankton pigments (in particular the chl *a*:carotenoid ratio) may change with depth due to photochromatic adaptation (Berner et al. 1989). Further, grazing in copepods is frequently linked to diurnal rhythms such as nocturnal grazing rather than to food concentrations (Landry et al. 1994). We believe that the (on average) 60% higher astaxanthin concentrations found in the *Calanus helgolandicus* night samples are indicative of nocturnal grazing. This view is consistent with previous studies showing higher body carotenoid concentrations in zooplankton around midnight (Hallegraeff et al. 1978, Kleppel et al. 1985).

Most importantly, zooplankton vertical migration may explain the lack of any clear vertical pattern of astaxanthin concentrations in *Calanus helgolandicus*. Given the small-scale sensitivity of zooplankton survival to surface/UV irradiance (Zagarese et al. 1998), synthesis of photoprotective pigments may be expected to depend strongly on the time-integrated exposure to high-energy irradiance. Although previous studies have provided contrasting results on the vertical migratory behaviour of *C. helgolandicus* (Williams 1985, Gowen et al. 1997, Iriogoien et al. 2004), the fact that *C. helgolandicus* was consistently absent from surface samples during the day but present at night indicates that at least part of the population in our study was vertically migrating. It could be speculated that the absence of *C. helgolandicus* from the surface and the slightly higher astaxanthin concentrations in the 4 m depth interval during the day were the result of photoprotective behaviour/pigment synthesis. Diurnal descent of surface individuals to intermediate depths also seems a likelier reason for the elevated diurnal changes of astaxanthin in the 8 m depth interval than selective predation by visual predators. Comparison of astaxanthin concentrations in *C. helgolandicus* (0.05 to 0.22 µg mg^{-1} DW) with those of conspicuously coloured copepods in ponds and rock pools (~2 to 10 µg mg^{-1} DW; Hairston 1978, Hessen & Sørensen 1990, Davenport et al. 2004), indicates that the range of astaxanthin concentrations in *C. hel-*
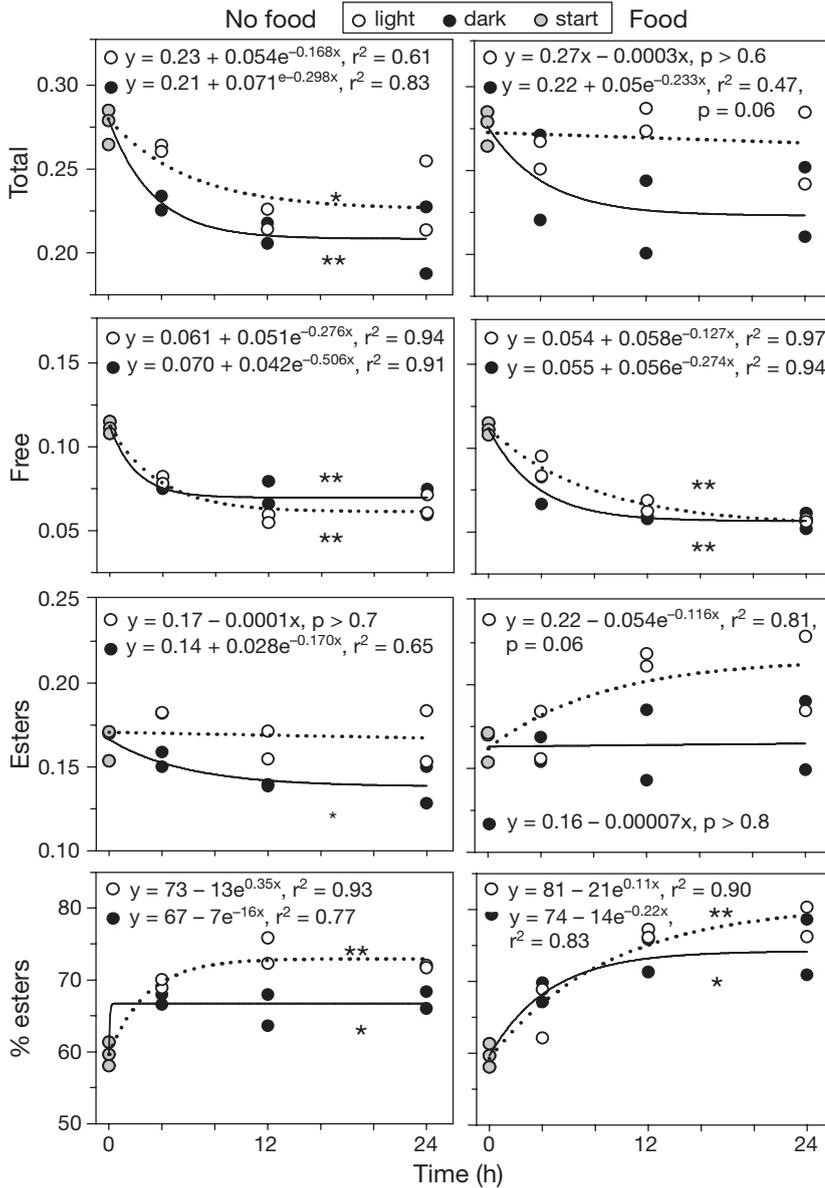
Fig. 4. *Calanus helgolandicus*. Astaxanthin (µg mg^{-1} DW) dynamics in the 2 × 2 factorial (food × light) experiment. No food, Food = treatments with unfiltered seawater from chl a maximum and GF/F-filtered seawater, respectively. Dotted and continuous lines = least-square regressions for treatments with and without light exposure, respectively. *p < 0.05, **p < 0.005

the underestimation of *C. helgolandicus* astaxanthin concentrations. This is probably also the reason why the initial samples of the onboard experiment, which were fixed immediately after sampling, had ~20 to 70 % higher astaxanthin concentrations than the field samples.

A more important result, however, was that the total astaxanthin concentrations were entirely determined by the dynamics of esterified astaxanthin. While the presence of food allowed copepods to maintain their initial level of esterified astaxanthin, the combined effect of food and light was to increase the concentration of astaxanthin esters. This was surprising, since newly synthesised astaxanthin may be expected to first become apparent as unesterified pigment. The reason for the decline of free astaxanthin in all treatments is unknown, but may be related to the experimental design. Our use of white fluorescent lamps exposed the copepods to a qualitatively different light regime than they experience in the field, including a significant portion of blue light that is absent at the depth (~42 m) of the chl a maximum (Jerlov 1976). Thus, it is possible that newly synthesised free astaxanthin was rapidly esterified as a specific response to the blue fraction of the emission spectrum. We may similarly speculate that the somewhat higher degree of astaxanthin esterification in the upper (≤12 m) water column during the day at Stn 35 was the result of a photoprotective response to high-energy irradiance (possibly, blue light).

However, the fact that the presence of food allowed copepods to sustain the levels of astaxanthin esters in treatments without light implies that astaxanthin esterification is *per se* a process independent of light. Similarly, other investigators have found that astaxanthin is deposited mainly as esters and not free astaxanthin in many crustaceans, particularly during ontogenetic development (Yamada et al. 1990, Petit et al. 1991, Dall et al. 1995), resulting in the predominance (~60 to 90 %) of astaxanthin esters in the adults of many crustaceans (Yamaguchi et al. 1983, Coral-Hinostroza & Bjerkeng 2002) including calanoid copepods (Foss et al. 1987, Juhl et al. 1996, Andersson et al. 2003,

golandicus is unlikely to produce predation-relevant differences in visual conspicuousness, given the generally turbid conditions of the North Sea.

The results of the 2 × 2 factorial experiment showed that astaxanthin production is a dynamic and rapid process. As reported in a previous study for the congeneric species *Calanus pacificus* (Juhl et al. 1996), astaxanthin degradation rates in *C. helgolandicus* may be particularly pronounced in starved individuals (~30 to 50 % d^{-1}). Thus, the prolonged handling time (~4 h) in our depth-profile experiment inevitably resulted in

Lotocka et al. 2004). Since the esterification of astaxanthin with fatty acids makes the pigment increasingly lipophilic (Kobayashi & Sakamoto 1999), the potential function of astaxanthin esters may be to improve the deposition and thus protection of storage lipids. This may be advantageous also in conditions when light is absent, e.g. during periods of diapause in deeper water layers, since reactive oxygen species responsible for lipid peroxidation are produced also during normal oxidative processes (Davies 1995).

In conclusion, we propose that the commonly accepted notion of a photoprotective role of astaxanthin, particularly against biologically harmful UV irradiance (Kouwenburg et al. 1999), needs to be refined. Although our results demonstrate that astaxanthin concentrations increase under light exposure, we suggest that photoprotection may represent but one case of a general beneficial antioxidant function of astaxanthin. The observation that copepods avoid surface waters during the daytime (Alonso et al. 2004, present study) and the occurrence of higher astaxanthin concentrations at night, when photoprotection is unnecessary, support this view.

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