

Carotenoid accumulation in copepods is related to lipid metabolism and reproduction rather than to UV-protection

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Abstract

Accumulation of carotenoid pigments in copepods has often been described as a plastic adaptation providing photoprotection against ultraviolet radiation (UVR). However, reports of seasonal carotenoid maxima in winter, when UVR is low, challenge the proposed driving role of UVR. Therefore, we here evaluate the mechanistic connection between UVR and the seasonal pattern of copepod carotenoid pigmentation. We assessed the carotenoids, fatty acid content and reproduction of *Leptodiatomus minutus* along with UVR exposure, water temperature, phytoplankton pigments, and fish predation in a boreal lake during 18 months covering two winter seasons. The predominant carotenoid astaxanthin occurred in free form as well as esterified with fatty acids. Mono- and diesters accounted for 62–93% of total astaxanthin and varied seasonally in close correlation with fatty acids. The seasonal variability in total astaxanthin content of the copepods was characterized by net accumulation in late fall of up to $0.034 \mu\text{g} (\text{mg dry mass})^{-1} \text{d}^{-1}$, which led to the mid-winter maximum of $3.89 \pm 0.31 \mu\text{g mg}^{-1}$. The two periods of net loss ($-0.018 \mu\text{g mg}^{-1} \text{d}^{-1}$ and $-0.021 \mu\text{g mg}^{-1} \text{d}^{-1}$) coincided with peaks of egg production in spring and summer leading to minimum astaxanthin content ($0.86 \pm 0.03 \mu\text{g mg}^{-1}$) in fall. This period was also characterized by the highest predation pressure by young-of-the-year fish. The results suggest that accumulation of astaxanthin in copepods is strongly related to lipid metabolism but not to UVR-photoprotection, and that seasonal changes of fatty acids and carotenoids are related to the reproduction cycle.

The red pigmentation of many zooplankton has long puzzled biologists, and various hypotheses have been offered to explain the phenomenon via proximate and ultimate causes (e.g., Brehm 1938). The red coloration of copepods is due to carotenoids, a large family of lipid-soluble pigments that are synthesized only in primary producers but may be either accumulated by zooplankton or biologically converted to other carotenoids, notably astaxanthin, which is the primary carotenoid among crustaceans (Matsuno 2001; Andersson et al. 2003; Rhodes 2006). Astaxanthin is a powerful antioxidant (McNulty et al. 2007) occurring both in free

form and esterified with fatty acids or associated with proteins (Cheesman et al. 1967; Matsuno 2001).

In zooplankton, carotenoid accumulation is a highly variable trait that has been linked to photoprotection against ultraviolet radiation (UVR) in field studies comparing lakes with differential UVR exposure and in experimental studies (Hairston 1976; Moeller et al. 2005; Hylander et al. 2009; Rautio and Tartarotti 2010; Sommaruga 2010). The underlying mechanism ascribing astaxanthin photoprotection properties involves the quenching of singlet oxygen ($^1\text{O}_2$) produced during UVR exposure rather than direct absorption or reflectance of the hazardous wavelengths (Krinsky 1979; Kobayashi and Sakamoto 1999). UV-exposed copepods at low water temperatures have especially been suggested to profit from increased carotenoid content to counteract the reduced efficiency of enzymatic UVR responses such as photoenzymatic repair at low water temperatures (Williamson et al. 2002; Hansson and Hylander 2009). Apart from UVR, carotenoids may protect copepods from other sources of oxidative stress, such as metal

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toxicants (Caramujo et al. 2012), and may also improve the immune defense (van Der Veen 2005) and reproductive output (Gorokhova et al. 2013) of the animals. Astaxanthin has also been shown to positively affect metabolic activity as well as egg production in copepods, supposedly due to its antioxidant properties (Gorokhova et al. 2013).

A possible link of copepod carotenoid pigmentation to lipid metabolism is indicated by the prevalence of carotenoids in lipid droplets in these animals (van Der Veen 2005). A large portion of astaxanthin may be esterified with fatty acids (Łotocka and Styczynska-Jurewicz 2001; Snoeijs and Häubner 2013), presumably to improve the antioxidant protection of storage lipids (Sommer et al. 2006). Accordingly, free astaxanthin may be incorporated into cell membranes, where it efficiently reduces lipid peroxidation while preserving membrane structure (McNulty et al. 2007). As astaxanthin must be accumulated or biologically converted from carotenoids in the diet, copepod pigmentation may be subject to seasonal shifts in the availability of an appropriate algal diet. Phytoplankton carotenoids that may serve as precursors for the synthesis of astaxanthin in copepods include β,β -carotene, lutein and zeaxanthin (Andersson et al. 2003; Rhodes 2006; Caramujo et al. 2012).

Carotenoid pigmentation may also be disadvantageous to copepods, as pigmented animals are generally more likely to be targeted by visual predators than unpigmented ones (Hairston 1979a; Gorokhova et al. 2013). Hence, copepods reduce their carotenoid content when exposed to predator cues (Hansson 2004; Hylander et al. 2012), and predation and UV radiation, acting in concert, are likely environmental factors affecting zooplankton pigmentation.

Although the environmental variables that potentially affect carotenoid accumulation in copepods exhibit considerable seasonal variations, no studies have explicitly focused on changes in copepod carotenoids in relation to the seasonally changing environment, including the ice-covered winter period. Several studies have shown a pronounced maximum in copepod carotenoid pigmentation in winter and a minimum during summer or early fall (Hairston 1979a; Hansson 2004; García et al. 2008; Ekvall et al. 2015), but without specifically addressing their controlling variables. Even when winter data are lacking, a steep decrease of carotenoid content in spring has been documented (Moeller et al. 2005). This pattern appears to contradict the hypothesis that carotenoid pigmentation is a direct response to UVR exposure.

Our goal in this study was to evaluate which factors are modulating carotenoid pigmentation in copepods by testing the hypotheses that variations in their astaxanthin content are related to: (1) seasonal changes in UVR exposure, with higher exposure inducing higher carotenoid accumulation; (2) water temperature, with the accumulation of carotenoids that act as antioxidants to compensate for reduced metabolic rate of repair enzymes at low temperatures; (3) the presence and abundance of dietary carotenoids that serve as precursors to copepod astaxanthin; (4) overall food availability and

quality, measured as the sum of carotenoid and chlorophyll pigments in the seston; (5) changes in the body content of fatty acids that may form esters with astaxanthin molecules; (6) reproduction and the transfer of carotenoids to eggs; and (7) predation pressure by fish that prey on the most pigmented copepods and/or force them to reduce carotenoid accumulation. We monitored a natural population of the copepod *Leptodiaptomus minutus* inhabiting a boreal shield lake during a period of 18 months including two winter seasons and large temporal variations in UVR exposure. A suite of environmental variables was assessed in order to test the above-mentioned hypotheses for winter pigmentation in a single, well-studied ecosystem.

Methods

Study site

Lake Simoncouche is a mesotrophic lake situated in the Laurentides Wildlife Reserve in Québec, Canada (lat. 48.23°N, long. 71.25°W; elevation 347 m a.s.l.). This dimictic, shallow lake ($Z_{\text{mean}} = 2.2$ m, $Z_{\text{max}} = 8$ m), has several inflows and one outflow, covers an area of 87 ha and is entirely surrounded by boreal forests. The ice cover typically forms towards the end of November and melts during the second half of April. In spite of the pronounced cold season, epilimnetic water temperatures rise to values above 20°C during July and August. Dissolved organic carbon concentrations range between 4.1 mg C L⁻¹ and 8.3 mg C L⁻¹ and the photic zone reaches the bottom. The crustacean zooplankton community of the lake principally consists of six copepod species (*L. minutus*, *Epischura lacustris*, *Aglaodiaptomus spatulocrenatus*, *Cyclops scutifer*, *Mesocyclops edax*, *Tropocyclops prasinus*) and five cladocerans (*Bosmina longirostris*, *Daphnia* spp., *Diabranosoma brachyurum*, *Holopedium gibberum*, *Leptodora kindtii*); furthermore *Chaoborus* sp. can be observed occasionally. The community is dominated by *L. minutus* that are present throughout the year forming two distinct cohorts (fall-winter and a spring-summer), and depending on the season contribute up to 87% (in winter) to the total crustacean zooplankton biomass.

Leptodiaptomus sampling and carotenoid analysis

Integrated samples of zooplankton were taken over the whole water column (0–6 m) at the deepest point of the lake on 23 occasions from 04 December 2011 to 07 May 2013. When the lake was ice-covered, sampling was conducted through a hole (diameter ca. 40 cm). Zooplankton were sampled by vertical net tows (diameter: 24 cm; mesh size: 50 μm) over the whole water column and kept in the dark during transport to the laboratory. Preliminary sampling had shown that adult *L. minutus* are homogeneously distributed in the water column during both the day and night irrespective of ice cover. Organisms were transferred to GF/F-filtered lake water using a 200 μm sieve and kept overnight at either 5°C (in winter) or 15°C (in summer) for gut evacuation. On the following day, adult *L. minutus* were individually picked from

CO₂-sedated zooplankton samples with a pair of forceps. If present, egg sacs were removed from female copepods. Between 100 and 200 individuals were collected for each replicate, with three replicates per analysis. On four dates from 13 June 2012 to 20 July 2012, adult *L. minutus* were rare, and the net pulls were dominated by copepodite CIII to CV stages. Because these stages are considerably smaller than adults, a larger number of individuals was required to obtain a sufficient amount of biomass. These samples typically contained between 400 and 800 copepodites and only very few adults; they were collected using a pipette, and were neither staged nor counted. On all other dates, only adults were collected. The animals were transferred into 1.5 mL-plastic tubes and stored at -80°C until freeze-drying.

Copepod carotenoids were analyzed by reversed-phase high-performance liquid chromatography (HPLC). Carotenoids were extracted from zooplankton in 90% (v/v) aqueous acetone, homogenized for 2 min (Caframo R2R1 tissue grinder, Warton, Ontario) on ice and then sonicated for three times 20 s on ice at 10 W using a rod sonicator (Microson XL2000, Misonix, Farmingdale, New York, U.S.A.). This protocol enabled optimal extraction of carotenoids from zooplankton samples (Rautio et al. 2009). The extracts were incubated overnight at -20°C under argon atmosphere, then centrifuged and filtered through 0.2 μm polytetrafluoroethylene membrane filters (VWR international, Mississauga, Ontario, Canada) and stored at 4°C in the dark under argon gas until HPLC analysis within 48 h. Fifty microliters were injected into an Accela 600 HPLC system (Thermo Scientific, Waltham, Massachusetts, U.S.A.) equipped with a Hypersil Gold C8 column (150 mm \times 4.6 mm, 3 μm particle size, Thermo Scientific) protected by a Hypersil Gold C8 guard column (10 mm \times 4 mm, 3 μm particle size, Thermo Scientific) using the HPLC protocol of Zapata et al. (2000). The run-time was 60 min for zooplankton. Peaks were detected by photodiode array spectroscopy (350–700 nm; slit width: 1 nm). Carotenoids were identified according to retention time and spectra of known standards and were quantified based on the absorbance chromatogram at 450 nm (Bonilla et al. 2005; Rautio et al. 2009). Mono- and diesters of astaxanthin were identified according to Snoeijs and Häubner (2013) by separating the first and second clusters of peaks.

UVR exposure and temperature

High-resolution vertical profiles of UVR and temperature were obtained with a PUV-2500 profiler radiometer (Biospherical Instruments, San Diego, California, U.S.A.) down the water column on the dates of zooplankton sampling. The PUV-2500 simultaneously measures UVR at six wavelengths (305, 313, 320, 340, 380, and 395 nm) together with broadband PAR (400–700 nm), water temperature and depth (via pressure). The UVR wavelengths cover the biologically relevant range of UV-B (280–320 nm) and UV-A (320–400 nm). The instrument recorded five measurements per second and was descended

downwards at approximately 0.1 m s^{-1} , thus obtaining ca. 50 data points per meter. Diffuse attenuation coefficients (K_d) of UVR in the water column were obtained from the slope of the linear regression of the natural logarithm of down-welling irradiance (E_d) vs. depth (Z), $\ln(E_{d(Z)}) = -K_d Z + c$, where the constant $c = \ln(E_{d(0^-)})$, with $E_{d(0^-)}$ being the irradiance just below the water surface. The 1%-penetration depth of UVR ($Z_{1\%}$) was calculated on ice-free dates as $Z_{1\%} = 4.605/K_d$ as in Schneider et al. (2012). When the lake was ice-covered, the radiometer was operated through a hole covered with an opaque cardboard square to avoid light passing through the hole influencing the measurement. To further minimize potential effects due to both the shadowing cover and the ice hole itself, values within 30 cm of the bottom of the ice cover were omitted from the K_d regression. The validity of this method was tested on 02 April 2013, where an aluminum arm was used to position the radiometer ca. 1 m away from the hole directly under the ice cover to allow for recording UVR close to the ice but without the effect of the hole. The values obtained on this date were within the range suggested by previous measurements (between 0% and 2% of surface radiation remaining under the ice in March and April), indicating that the error due to operating the radiometer directly under the hole was negligible. The attenuation due to ice and snow cover was estimated as:

$$K_{\text{ice}} = 1 - (E_{d(\text{ice}^-)} / E_{d(0)}),$$

where $E_{d(\text{ice}^-)}$ is the irradiance at the bottom of the ice cover and $E_{d(0)}$ is the surface irradiance measured in air. Because $E_{d(\text{ice}^-)}$ could not be measured directly, it was extrapolated from the above-mentioned linear regression,

$$\ln(E_{d(\text{ice}^-)}) = -K_d Z_{\text{ice}^-} + c,$$

where Z_{ice^-} is the depth of the bottom of the ice cover and c is the constant obtained from calculating K_d .

Irradiance at a certain depth depends on both the surface irradiance and the attenuation by water, ice and snow. The latter can be reliably determined independent of weather conditions, but incident irradiance strongly depends on the precise time of day as well as on cloud cover and air humidity during the measurement. In order to obtain an estimate of the UVR exposure representative for the general seasonal pattern but independent of short-term meteorological fluctuations, we derived the incident surface irradiance $E_{d(0)}$ at noon on each sampling date from the model FASTRT (Engelsen and Kylling 2005; available at <http://zardoz.nilu.no/~olaeng/fastrt/fastrt.html>) for the coordinates of Lake Simoncouche. The model parameters were set to cloudless sky, 25 km visibility and zero surface albedo. Since the seasonal patterns of irradiance and attenuation were very similar for all wavelengths measured, we used the irradiance at 380 nm as a proxy for all UVR because wavelengths shorter than that were undetectable on some dates under the ice.

On dates where the lake was ice-covered, the radiation remaining below the ice was calculated as:

$$E_{d(\text{ice}^-)} = E_{d(0)}(1 - K_{\text{ice}}).$$

The radiation remaining at 1 m depth was then subject to further attenuation in the water column and was calculated as:

$$E_{d(Z)} = E_{d(\text{ice}^-)} e^{-K_d \Delta Z},$$

where $\Delta Z = Z - Z_{\text{ice}^-}$, i.e., the distance between the bottom of the ice layer and 1 m depth. During the ice-free period, underwater irradiance was directly calculated as:

$$E_{d(Z)} = E_{d(0)} e^{-K_d Z}.$$

Abundance of dietary pigments and copepod fatty acids

Water for the analysis of phytoplankton pigments was collected at regular depth intervals (1 m or 1.5 m) including a sub-surface sample using a 2 L cylindrical water sampler equipped with a messenger-controlled closing mechanism (Limnos Ltd., Turku, Finland). The water was prefiltered through a 50 μm Nitex screen to exclude zooplankton, and kept cool and dark until filtration onto GF/F filters (24 mm; three replicates of each layer) in the laboratory on the same day. The filtration volume ranged from 0.1 L to 0.5 L depending on the abundance of suspended material in the water, which was always visible as a brownish film on the filter. The filters were wrapped in aluminum foil and stored in airtight plastic bags at -80°C until freeze-drying, after which they were stored at -50°C . Carotenoids and chlorophylls were extracted from frozen seston filters in 95% (v/v) aqueous methanol for 30 min on ice and sonicated as described above (Zapata et al. 2000). Pigments were analyzed by HPLC as described above for copepod carotenoids except that runtime was only 40 min and a fluorescence detector (excitation: 440 nm; emission: 650 nm) was used in addition to the photodiode array. The quantification of carotenoids was based on the absorbance chromatogram at 450 nm, while chlorophylls were quantified from the fluorescence chromatogram using calibration curves based on known standard concentrations (Bonilla et al. 2005; Rautio et al. 2009). All seston pigments were expressed as $\mu\text{g L}^{-1}$.

Lipids were extracted from copepod samples in chloroform-methanol mixture following Heissenberger et al. (2010). The lipid extracts were then solubilized in toluene, and H_2SO_4 -methanol (1% v/v) was added to promote transesterification at 50°C ; the resulting fatty acid methyl esters (FAMES) were separated from non-FAME components by addition of KHCO_3 -water (2% v/v) and hexane (modified from Heissenberger et al. 2010).

FAMES were analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A chromatograph (Agilent Technologies, Santa Clara, California, U. S. A.) equipped with an Agilent 5975C mass spectrometer with

triple-axis detector and an Agilent J&W DB-23 column (60 m length, 0.25 mm inner diameter, 0.15 μm film thickness). Helium was used as the carrier gas (flow rate 1 mL min^{-1} with electronic pressure control) and the temperature ramp was as follows: 70°C for 1.5 min followed by an increase of $20^\circ\text{C min}^{-1}$ until 110°C , an increase of $12.5^\circ\text{C min}^{-1}$ until 160°C , and an increase of $2.5^\circ\text{C min}^{-1}$ until the final temperature of 230°C , which was maintained for 6.5 min resulting in 42 min total run time. The GC was equipped with a temperature-programmable injector and an autosampler. FAMES were identified by retention time and ion composition and were quantified from the peak area of the most abundant ion out of the four ions recorded (m/z 74, 79, 81, and 87) vs. an internal standard (nonadecanoic acid) using calibration curves based on known standard concentrations.

Copepod egg production and fish predation pressure

The reproductive effort of *L. minutus* was estimated by the number of eggs per female copepod (egg ratio), which has been assessed in Lake Simoncouche from 19 May 2011 to 23 May 2012 (G. Grosbois, unpubl. data). Sampling frequency was weekly during the open-water period and bi-weekly when the lake was ice-covered. The quantitative samples (12–30 L), collected at regular intervals from the whole water column, were conserved in formaldehyde (4% final concentration) until counting. Entire samples or aliquots (typically 50%) were counted on an inverted microscope ($\times 50$ magnification; Axio Observer.A1, Zeiss, Jena, Germany). Females were identified according to the morphology of their P5. Eggs were identified either as attached to *L. minutus* females or as free detached eggs. Typically, between 200 and 500 crustacean individuals (including juvenile but not larval stages) were counted in each sample. The high sampling frequency allowed for enough data points to represent the complete seasonal variability, and to minimize the effects of spatial variability. The values were averaged for each calendar month to equalize potential shifts in the seasonal timing among years.

Lake Simoncouche is inhabited by the pelagic brook trout, *Salvelinus fontinalis*, the bottom feeding white sucker, *Catostomus commersoni*, and several species of minnows: *Couesius plumbeus*, *Semotilus atromaculatus*, *Semotilus margarita*, *Notropis atherinoides* and *Notropis hudsonius*. To estimate seasonal differences in visual predation pressure on copepods we assumed that this predation is exerted mainly by young-of-the-year (YOY) of brook trout. This assumption was based on stable isotope signatures obtained in summer 2011 that revealed small differences in the $\delta^{15}\text{N}$ values (\pm standard deviation) between the minnows ($7.2 \pm 0.4\text{‰}$) as compared to *L. minutus* ($5.6 \pm 0.2\text{‰}$), indicating that minnows are not feeding extensively on this copepod (when accounting for a trophic fractionation of $+3.4\text{‰}$; Post 2002); on the contrary, brook trout $\delta^{15}\text{N}$ was $8.4 \pm 0.5\text{‰}$ and thus in the expected range for it to be a potential predator on *L. minutus* (G. Grosbois, unpubl. data). Prey consumption per unit weight of

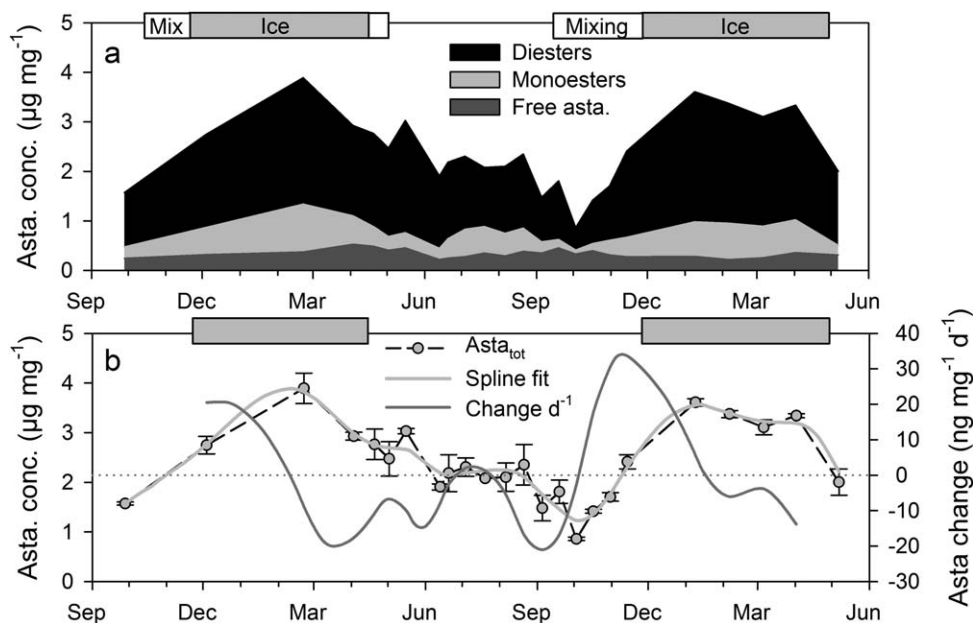


Fig. 1. Seasonal variation of copepod carotenoid concentration. (a) Astaxanthin fractions. (b) Total astaxanthin (\pm SE), spline fitting and rate of change. Periods of ice cover and mixing are indicated by grey and white bars, respectively.

brook trout is limited by water temperature (highest between 15°C and 22°C) and continually decreases as the fish grow (Hartman and Sweka 2001). We applied the Hartman and Sweka (2001) model for brook trout maximum consumption rate to Lake Simoncouche using the water temperature measured at 1 m depth combined with literature data on the temporal development of YOY individual weight as well as biomass (Hunt 1966) and scaled the resulting seasonal pattern so that maximum consumption equaled one. Potential shifts in the precise timing of YOY development are unlikely to impact the general pattern of consumption, as both YOY weight and biomass were continually increasing throughout the year (Hunt 1966).

Data analysis

We evaluated copepod carotenoid pigmentation both as astaxanthin content ($Asta_{tot}$; $\mu\text{g mg}^{-1}$) and as the rate of change ($Asta$ change; $\mu\text{g mg}^{-1} \text{d}^{-1}$). The latter variable was introduced in order to assess how the copepods respond to environmental drivers by increasing or reducing their carotenoid content. Our interest was in the general seasonal pattern rather than short-term fluctuations, therefore we based this estimate on a penalized cubic regression spline fitted on copepod astaxanthin concentration vs. date ($R^2 = 0.78$). The rate of change was then calculated as the first derivative of the regression spline on each sampling date. The spline was modeled as a straight line at its end points, and therefore the first and the last date of the fitted period (28 September 2011 and 7 May 2013) were not used for the rate of change.

Pairwise correlations were calculated between *Leptodiaptomus* astaxanthin (mono- and diesters, free astaxanthin, total

content and rate of change) and environmental variables. We applied multiple linear regression (MLR) analysis to identify the best explanatory variables (temperature, diet, fatty acids, reproduction, and predation) for copepod astaxanthin concentration and for the rate of change in copepod astaxanthin. UV radiation was excluded from the analysis due to its highly significant negative correlation with $Asta_{tot}$ ($r = -0.81$, $p < 0.001$), which does not correspond to any ecologically meaningful explanation for astaxanthin accumulation. The astaxanthin precursors present in the seston (β , β -carotene, lutein, and zeaxanthin) were highly correlated with water temperature ($r = 0.91$, $p < 0.001$). These pigments were therefore not considered in the multiple regression analysis. Instead, we used the sum of total seston carotenoids and chlorophylls as an indicator of general food abundance (correlation with temperature: $r = 0.66$). The assumptions of normality and homoscedasticity were evaluated based on scatterplots; egg ratio values were $\log(X + 0.1)$ -transformed. The variables included in the analysis were thus: water temperature at 1 m (Temp), sum of seston carotenoids and chlorophylls (Diet), copepod total fatty acid concentration (FA_{tot}), the egg ratio (Eggs) and YOY fish consumption (YOY). The best models were selected based on the lowest Akaike Information Criterion corrected for small sample sizes (AIC_c ; Burnham and Anderson 2002). For ease of interpretation, the results are presented as ΔAIC_c , which is the difference between a given AIC_c value and the best model's (i.e., lowest) AIC_c value. To address model uncertainty, the relative variable importance was calculated as the sum of the Akaike weights of all models including a given variable;

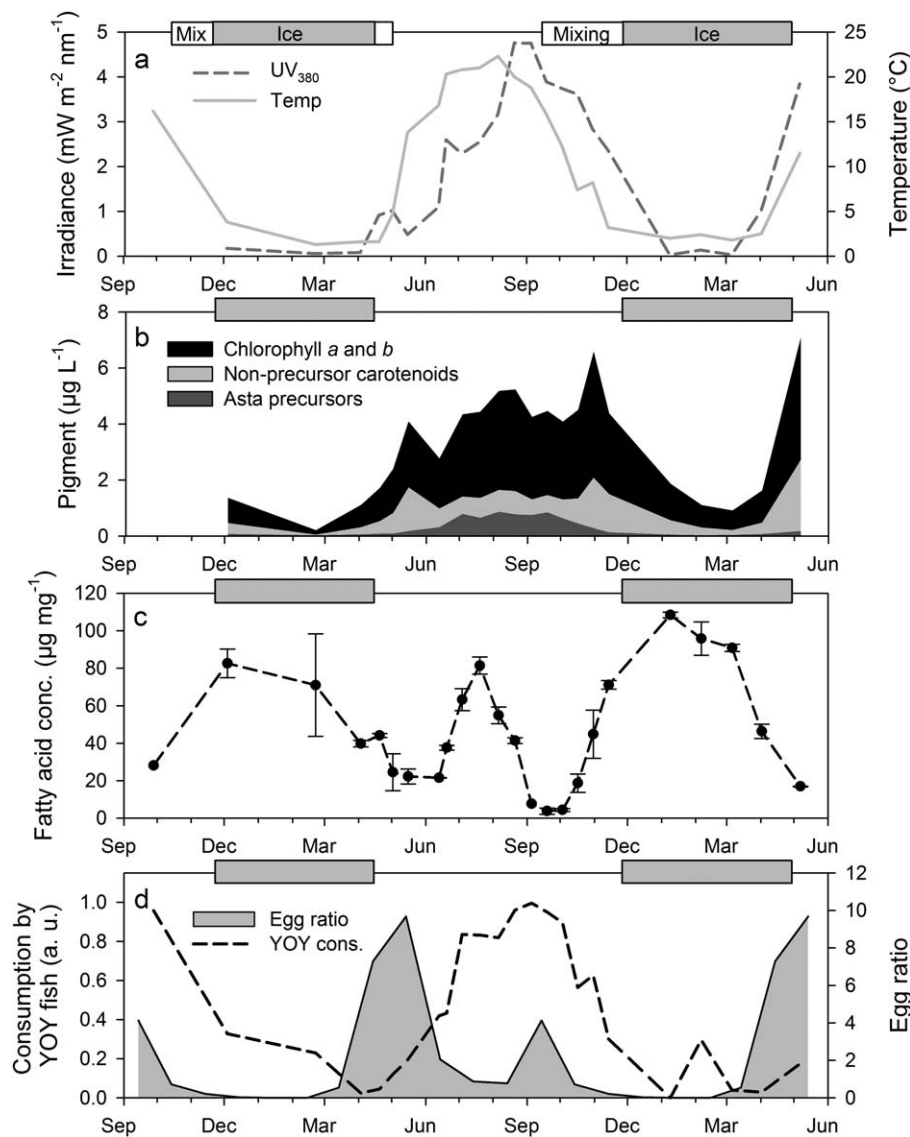


Fig. 2. Seasonal variation of factors putatively modulating copepod carotenoid pigmentation. (a) Irradiance at 380 nm at 1 m depth (UV_{380}) and water temperature at 1 m (Temp). (b) Pigment concentration in the seston. (c) Total fatty acid concentration (\pm SE) in *L. minutus*. (d) Egg ratio (eggs per female; monthly averages) and consumption by young-of-the-year brook trout as a proxy for fish predation (YOY cons.). Error bars in (c) indicate the standard error of the mean.

this value expresses the probability that a variable is part of the actual best model among all the considered models (Burnham and Anderson 2002). Spline fitting, pairwise correlation and multiple regression analyses were carried out using the software JMP version 10.0 (SAS Institute, Cary, North Carolina, U.S.A.).

Results

The total concentration of copepod astaxanthin reached its maximum (\pm SE) in mid-winter ($3.89 \pm 0.31 \mu\text{g mg}^{-1}$ on 22 February 2012) and then decreased throughout spring and summer until a minimum was reached in late summer/

early fall ($0.86 \pm 0.03 \mu\text{g mg}^{-1}$ on 03 October 2012; Fig. 1a). During the following three months, copepod astaxanthin content increased fourfold to reach a winter maximum of $3.61 \pm 0.08 \mu\text{g mg}^{-1}$ on 09 January 2013. Copepod carotenoid concentration was significantly higher when the lake was ice-covered as compared to the open water period (*t*-test, $p < 0.001$). This pattern was equally pronounced when carotenoids were expressed as ng per individual copepod (ng ind^{-1}) (*t*-test, $p < 0.001$) and both measures of carotenoids were strongly correlated ($r = 0.93$, $p < 0.001$). The rate of change in $Asta_{\text{tot}}$ was highest in late fall (maximum accumulation of $33.6 \text{ ng mg}^{-1} \text{ d}^{-1}$ on 14 November 2012) and was strongly negative in March/April as well as in September

Table 1. Correlation with potential explanatory variables of total astaxanthin and rate of change of this pigment in the copepod *Leptodiaptomus minutus* sampled in Lake Simoncouche. UV₃₈₀, irradiance of UVR at 380 nm at 1 m depth; Temperature, water temperature at 1 m depth; Precursors, sum of potential astaxanthin precursors (β,β -carotene, lutein and zeaxanthin) in the lake seston; Food, the sum of carotenoid and chlorophyll pigments in the seston; YOY consumption, potential feeding pressure by young-of-the-year fish; Eggs, $\log(X + 0.1)$ -transformed egg ratio; FA_{tot}, total fatty acid concentration in *L. minutus*. r , correlation coefficient; N , number of samples.

Variable	Total Astaxanthin			Astaxanthin change		
	r	N	p	r	N	p
UV ₃₈₀	-0.81	23	<0.001	-0.02	22	0.937
Temperature	-0.60	24	0.002	-0.25	22	0.271
Precursors	-0.67	22	0.001	-0.20	21	0.383
Food	-0.71	22	<0.001	0.28	21	0.211
YOY consumption	-0.73	24	<0.001	-0.05	22	0.824
Eggs	-0.31	24	0.140	-0.50	22	0.017
FA _{tot}	0.69	24	<0.001	0.34	22	0.124

($-17.6 \text{ ng mg}^{-1} \text{ d}^{-1}$ and $-21.0 \text{ ng mg}^{-1} \text{ d}^{-1}$, respectively; Fig. 1b). A third but smaller negative peak in Asta change occurred between two sampling dates in May/June and coincided with the replacement of overwintering adults by copepodites of the next generation.

The carotenoids in copepods were comprised mainly (> 99%) of astaxanthin in three forms: free astaxanthin, monoesters and diesters, complemented by traces of alloxanthin and β,β -carotene. Overall, free astaxanthin and monoesters accounted for $14 \pm 4\%$ and $18 \pm 9\%$ (mean \pm SD), respectively, while diesters represented $68 \pm 25\%$ of total astaxanthin (Asta_{tot}; hereafter used synonymously for total carotenoids per dry mass) in *L. minutus* (Fig. 1a). At any time of the year, between 62% and 93% of astaxanthin was esterified. The three fractions described differential seasonal patterns, with free astaxanthin showing only small seasonal changes as compared to the esterified fractions (Fig. 1a). Monoesters and diesters were positively correlated with each other ($r = 0.78$, $p < 0.001$) and with Asta_{tot} ($r = 0.86$ and $r = 0.98$, respectively; both $p < 0.001$), while no such correlation was found involving free astaxanthin ($p > 0.5$).

All environmental variables showed strong seasonal variation. The lake was ice-covered from 28 November 2011 to 18 April 2012 and from 20 November 2012 to 03 May 2013. Water transparency to UVR was slightly lower in spring ($K_{d(380 \text{ nm})} = 7.3 \text{ m}^{-1}$ on 16 May 2012) than in late summer and fall ($K_{d(380 \text{ nm})} = 4.6 \text{ m}^{-1}$ on 17 October 2012). Despite these seasonal differences, Lake Simoncouche was characterized as a low-UVR environment throughout the year. During the open-water period, UV-B (320 nm) was attenuated

within the first 40 cm ($Z_{1\%}$ between 23 cm and 39 cm) and longer wavelength UV-A (380 nm) radiation did not penetrate deeper than 1 m ($Z_{1\%}$ at 63–99 cm) into the water column. Estimated underwater UVR irradiance reached its maximum in August–September but was strongly reduced when the lake was ice-covered (Fig. 2a). Likewise, water temperature and seston pigment concentration were low during winter but increased rapidly after ice breakup (Fig. 2a). Phytoplankton pigments were dominated by chlorophyll *a*, followed by fucoxanthin, zeaxanthin, alloxanthin, chlorophyll *b*, violaxanthin, β,β -carotene, diadinoxanthin, lutein, and the occasionally present 9'-*cis*-neoxanthin. Fucoxanthin and violaxanthin showed two distinct seasonal maxima in May and October–November, while alloxanthin and diadinoxanthin peaked at the same time but had an additional maximum in July–August. The putative astaxanthin precursors, lutein, β,β -carotene and zeaxanthin, were most abundant between the two peaks of fucoxanthin; they had a broad maximum from June to October following the general seasonal pattern of epilimnetic water temperature ($r = 0.88$). The concentration of Chl *a* was positively correlated with the sum of all carotenoids ($r = 0.92$), with a minimum during winter and maxima in spring, summer and during the fall overturn (Fig. 2b).

The concentration of total fatty acids (FA_{tot}) in copepods was highest in winter but showed an additional maximum in July–August (range 3.7–108.5 $\mu\text{g mg}^{-1}$; Fig. 2c). This peak partly coincided with the presence of juvenile stages in the samples from 13 June 2012 to 20 July 2012. The composition of FAs was constant throughout most of the year with saturated FAs (SAFAs) contributing on average 40%, monounsaturated FAs (MUFAs) 14% and polyunsaturated FAs (PUFAs) 46% of FA_{tot}.

There was a strong seasonal pattern of reproductive output by the *Leptodiaptomus* population, with a complete absence of eggs from December to February. The main reproduction peak occurred in April and May (9.7 eggs per female *L. minutus*), with a lower secondary peak in September (4.1 eggs per female; Fig. 2d).

The modelled consumption rate by YOY fish resulted in a seasonal pattern that showed a maximum from late August to early October and was correlated with water temperature ($r = 0.78$, $p < 0.001$; Fig. 2d). This was consistent with literature reports of increased fish predation on zooplankton in late summer/early fall in temperate lakes (Warren et al. 1986; Hansson 2004; Sirois et al. 2011).

Asta_{tot} was negatively correlated with both UVR exposure at 380 nm ($r = -0.81$, $p < 0.001$) and water temperature ($r = -0.60$, $p = 0.002$; Table 1). It was also negatively correlated with all potential astaxanthin precursors ($r < -0.57$, $p < 0.01$) and with the sum of these precursors (-0.67 , $p < 0.001$) as well as with total phytoplankton pigments ($r = -0.71$, $p < 0.001$) and YOY consumption rate (-0.73 , $p < 0.001$). Asta_{tot} was positively correlated with total fatty

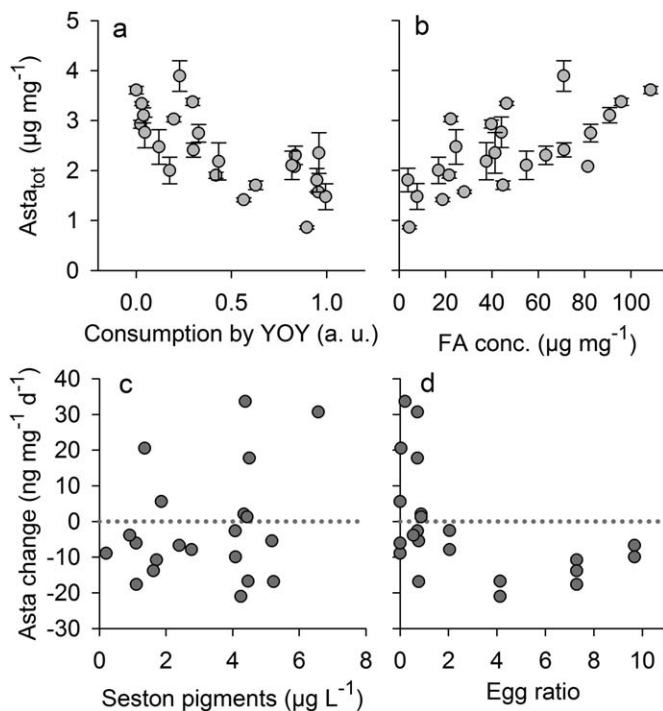


Fig. 3. Total astaxanthin concentration in copepods ($Asta_{tot} \pm SE$; a, b) and the rate of change in $Asta_{tot}$ (c, d) relative to selected explanatory variables: (a) Consumption by YOY brook trout, (b) fatty acid concentration in copepods, (c) total phytoplankton pigments in the seston, (d) egg ratio. In (c) and (d), zero daily change is indicated by a dotted grey line.

acids ($r = 0.69$, $p < 0.001$) and unrelated to the egg ratio ($p > 0.1$). The correlation with FA_{tot} was due to the esterified fractions (monoesters: $r = 0.83$; diesters: $r = 0.66$) rather than free astaxanthin ($r = -0.42$). These relationships were very similar when the four dates with predominantly juvenile individuals were excluded from the analysis (free Asta: -0.42 ; monoesters: 0.81 ; diesters: 0.73); this was tested to verify that in spite of the summer fatty acid peak during this period and potential physiological differences among life stages, inclusion of these samples did not largely alter these relationships. The rate of change in $Asta_{tot}$ was negatively correlated with the *Leptodiptomus* egg ratio ($r = -0.50$, $p = 0.017$) but was unrelated to other environmental variables (Table 1).

MLR analysis of the relationship between environmental variables and the variation of copepod astaxanthin content showed that three models could be selected according to their AIC_c values ($\Delta AIC_c < 2$, which is the threshold identifying potential best models; Burnham and Anderson 2002). The main variables contributing to these three models were copepod fatty acid content and YOY consumption that together accounted for 72% of total variation in copepod astaxanthin content (Table 2; Fig. 3a,b). These two variables had the highest relative variable importance (RVI) of 0.95

and 0.84, respectively, indicating that they were likely part of the true best model for $Asta_{tot}$ (i.e., based on the variables tested). RVI was intermediate for phytoplankton pigments (0.66), while egg ratio and temperature were relatively unimportant (Table 2).

For Asta change, MLR revealed four models with $\Delta AIC_c < 2$ (Table 2). Within those, the three best models had very similar AIC_c values ($\Delta AIC_c < 1$) and Akaike weights (between 0.20 and 0.26, summing up to 0.70). The two most important variables were phytoplankton pigments and egg ratio (RVI of 1.00 and 0.84), which occurred together in the three best models in combination with YOY consumption and/or water temperature (RVI > 0.5 each; Fig. 3c,d), while copepod fatty acid concentration was of low importance for variation in Asta change.

Discussion

Our results provide evidence of a strong link between copepod astaxanthin and lipid content as well as reproduction of the animals, with additional effects of temperature, diet and fish predation. The astaxanthin pigmentation of copepods in this boreal lake ecosystem changed markedly with season, but the exposure to solar UV radiation had no apparent effect on copepod carotenoids in this low-UVR lake. Astaxanthin pigmentation was most pronounced in early- and mid-winter when the lake was ice-covered and when there was no UV radiation threat. Instead, the elevated astaxanthin concentration in copepods during winter was related to the high concentration of lipids in the copepods and to low predation pressure. Moreover, the periods of most pronounced astaxanthin loss rates overlapped with the timing of supposed carotenoid transfer to the eggs, while the overall high abundance of phytoplankton pigments in the water column favored astaxanthin accumulation.

The total carotenoid concentration in *L. minutus* in Lake Simoncouche was well within the range previously described for this species (Moeller et al. 2005; Rautio et al. 2009), and large seasonal differences in copepod carotenoid content with maximum values in winter have also been observed elsewhere (Hairston 1979a; Hansson 2004; Ekvall et al. 2015). Previous studies have tended to emphasize the photo-protective role of carotenoids, which has been well demonstrated. For example Ringelberg et al. (1981) has shown that carotenoid-rich copepods tolerate higher levels of UVR compared to unpigmented individuals. When experimentally exposed to UV-A radiation, copepods increased their carotenoid content relative to visible light only treatments (Hylander et al. 2009). Similarly, spatial comparisons among highly UV-exposed lakes have revealed that copepods adapt their carotenoid content according to UVR exposure (Hylander et al. 2009; Sommaruga 2010). Our results, however, suggest that the seasonal pattern in copepod carotenoid concentration in Lake Simoncouche may not be explained

Table 2. Results of the best multiple linear regression models to estimate total astaxanthin ($Asta_{tot}$; $\mu\text{g mg}^{-1}$) and the rate of change of astaxanthin ($Asta$ change; $\text{ng mg}^{-1} \text{d}^{-1}$) in *L. minutus* in Lake Simoncouche, according to lowest AIC_c . The predictor variables considered in the multiple regression were as described in Table 1. Relative variable importance (RVI) below each predictor variable, p -value and semipartial R^2 (SR^2) below the standardized regression coefficient (Std β), total R^2 and adjusted R^2 , mean square error (MSE), ΔAIC_c and Akaike weight (w_i) are shown. The number of observations was 22 for $Asta_{tot}$ and 21 for $Asta$ change.

Model		Temp	Food	YOY cons.	Eggs	FA_{tot}	R^2 (adj. R^2)	MSE	ΔAIC_c	w_i
$Asta_{tot}$	RVI	0.24	0.66	0.84	0.28	0.95				
A1	Std β		-0.29	-0.39		0.41	0.76	0.1634	0.00	0.27
	p		0.0780	0.0146		0.0056	(0.72)			
	SR^2		0.05	0.10		0.13				
A2	Std β			-0.53		0.49	0.72	0.1848	0.51	0.21
	p			0.0006		0.0013	(0.69)			
	SR^2			0.25		0.21				
A3	Std β	0.27	-0.35	-0.56		0.43	0.78	0.1571	1.74	0.11
	p	0.2081	0.0401	0.0098		0.0041	(0.73)			
	SR^2	0.02	0.06	0.11		0.14				
A4	Std β		-0.47		0.38	0.74	0.73	0.1828	2.48	0.08
	p		0.0032		0.0459	0.0012	(0.69)			
	SR^3		0.17		0.07	0.22				
A5	Std β		-0.32	-0.30	0.20	0.57	0.78	0.1627	2.50	0.08
	p		0.0572	0.0901	0.3150	0.0119	(0.72)			
	SR^2		0.05	0.04	0.01	0.10				
$Asta$ change	RVI	0.69	1.00	0.54	0.84	0.28				
B1	Std β		1.04	-0.84	-0.69		0.69	88.55	0.00	0.26
	p		0.0002	0.0010	0.0001		(0.63)			
	SR^2		0.43	0.29	0.44					
B2	Std β	-0.78	0.91		-0.45		0.68	89.06	0.12	0.24
	p	0.0010	0.0002		0.0056		(0.63)			
	SR^2	0.29	0.42		0.19					
B3	Std β	-0.45	1.08	-0.50	-0.57		0.73	79.68	0.51	0.20
	p	0.1083	<0.0001	0.1024	0.0017		(0.67)			
	SR^2	0.05	0.46	0.05	0.24					
B4	Std β	-0.82	1.06			0.46	0.66	94.72	1.42	0.13
	p	0.0008	<0.0001			0.0098	(0.60)			
	SR^3	0.32	0.53			0.17				
B5	Std β	1.04	-0.91		-0.80	-0.15	0.71	88.05	2.61	0.07
	p	0.0002	0.0016		0.0027	0.5398	(0.63)			
	SR^2	0.43	0.27		0.24	0.01				

by photoprotection alone since the astaxanthin content of the copepods in our study was inversely correlated with UVR and the rate of change in astaxanthin content was statistically unrelated to underwater UV irradiance. The results are in agreement with reports of high carotenoid content in copepods in low-UVR environments, such as the Baltic Sea or extremely turbid salt lakes (Sommer et al. 2006; Schneider et al. 2012), further suggesting that in low-UVR systems photoprotection is not the primary function of carotenoid pigmentation. Instead, carotenoids may also provide other benefits than photoprotection against UVR, and may be

related to physiological processes that are potentially affected by other environmental factors.

Low temperatures and a shortage of food in winter are physiologically demanding for copepods and could lead to oxidative stress, and in such situations high astaxanthin concentration may reduce this stress by acting as an antioxidant. Astaxanthin esters are thought to be allocated towards lipid storage in copepods (Sommer et al. 2006) and have been hypothesized to provide antioxidant protection to storage lipids in winter (Snoeijs and Häubner 2013). It has also been speculated that astaxanthin may serve as a

physiological replacement for molecular oxygen, allowing for rapid utilization of energy reserves (Łotocka 2004). A similar physiological role of astaxanthin has been suggested by Gorokhova et al. (2013), whose results support the metabolic stimulation hypothesis. These authors propose that the antioxidant protective capacity of astaxanthin allows copepods to up-regulate metabolic processes that would otherwise cause damaging oxidative stress. Furthermore, free astaxanthin may be incorporated into cell membranes to prevent peroxidation of PUFAs, which are especially important for membrane fluidity in cold environments (McNulty et al. 2007; Caramujo et al. 2012). Such a division of physiological roles among different fractions of astaxanthin is supported by our results. The relatively constant levels of free astaxanthin suggest its continuous presence in membranes in low concentration; in contrast, the strong seasonal variability of astaxanthin mono- and diesters was consistent with their putative role in antioxidant protection of storage lipids.

Fatty acid concentrations in the *L. minutus* population of Lake Simoncouche showed two peaks, one in January–February and another in July–August, corresponding to the winter and summer generations of this bivoltine population. However, the lipid peak in summer was paralleled solely by astaxanthin monoesters, whereas the accumulation of the more abundant diesters was limited to winter. Thus, the proposed link between astaxanthin and lipid reserves appear to be altered by differences between the two cohorts, such as growth rate, water temperature, predation pressure or other factors yet to be elucidated. The negative correlation between free astaxanthin and fatty acids suggests that some of the free astaxanthin molecules become esterified in the course of lipid accumulation. Interestingly, free astaxanthin was most prevalent during periods of egg production ($r = 0.57$, $p = 0.004$ with untransformed egg ratio). This may be due to the fact that astaxanthin is transferred to eggs in its free form rather than as esters (Łotocka 2004). These observations further underline the dynamic use and reallocation of astaxanthin due to varying physiological demands within the copepods in different seasons.

We observed two distinct periods of net loss of astaxanthin in late winter and late summer, coinciding with the peaks in the egg carrying ratio of the winter and summer generations, respectively. Accordingly, the net accumulation of astaxanthin only occurred when the egg ratio was low, showing that astaxanthin accumulation and egg production were temporally separated, suggesting that the build-up of astaxanthin reserves was counteracted by investment in reproduction. A less pronounced period of loss occurred in May–June and was most likely related to the replacement of overwintering adults by juveniles of the summer generation, which had lower astaxanthin content. Winter maxima in carotenoid content preceding maximum egg production have been explained by the transfer of carotenoids to the offspring (Hairston 1979b), and copepod astaxanthin concentration has been shown to be positively linked to reproductive

output in marine copepods (Gorokhova et al. 2013). In the course of reproduction, a loss of carotenoid and lipid content in adult copepods should be expected due to the transfer of both astaxanthin and fatty acids to the eggs (Hairston 1979b; Łotocka 2004). Copepod nauplii might benefit from carotenoid reserves via photoprotection (allowing them to stay in warmer surface waters) or via metabolic stimulation (Hairston 1979b; Łotocka 2004).

To be able to accumulate astaxanthin for lipid metabolism and reproduction, copepods first need to obtain the required phytoplankton precursors from their diet. We therefore expected food supply to play a role in copepod carotenoid pigmentation. The potential astaxanthin precursors present in the seston, β,β -carotene, lutein and zeaxanthin (Matsuno 2001; Andersson et al. 2003; Rhodes 2006), showed weak negative correlations with the astaxanthin accumulation rate in copepods indicating that these compounds were not directly controlling astaxanthin accumulation. The substantial increase in astaxanthin content during late fall suggests that astaxanthin precursor accumulation has occurred before the phytoplankton community declined in early winter. Comparable prewinter accumulation of reserves and subsequent reliance thereon have been suggested for copepod storage lipids (Rautio et al. 2011). The observed positive relationship between astaxanthin rate of change and phytoplankton pigments (measured as the sum of carotenoids and chlorophylls in the seston) likely reflects the overall benefits of high food abundance. Furthermore, as astaxanthin in copepods was closely related to their fatty acid content, our results suggest that astaxanthin was accumulated together with lipids during periods of abundant, high-quality food. It thus appears more plausible that carotenoid uptake was coupled to lipid accumulation rather than directly to food concentration.

The seasonal variations of food availability and reproductive effort were further modified by ambient water temperature. Our initial hypothesis that copepod carotenoid concentration would be negatively related to water temperature was based on the assumption of a primarily photoprotective role of the pigments: the enhanced efficiency of enzymatic processes such as photo-repair at higher temperatures would reduce the need for nonenzymatic antioxidants such as carotenoids (Williamson et al. 2002; Häder et al. 2015). Although we found no indication that UVR poses a significant threat to planktonic copepods in Lake Simoncouche, temperature was negatively correlated with copepod astaxanthin concentration, with warmer environments ($> 15^{\circ}\text{C}$) generally leading to a reduction in astaxanthin content. However, astaxanthin reduction also occurred at low temperatures $< 3^{\circ}\text{C}$, while net accumulation was limited to the intermediate temperature range between 3°C and 10°C . The unimodal pattern emerges from the two main periods of astaxanthin loss in winter and late summer, and from the net accumulation being limited to late fall and the onset of

winter, and can be explained by the ecological need of the developing fall cohort to acquire the resources they need to survive and reproduce during winter combined with investment into reproduction as discussed above.

While there are several physiological benefits associated with the accumulation of carotenoids, the primary reason to avoid them is increased detectability by visual predators. Pigmented copepods are at a higher risk of visual predation (Hairston 1979a; Gorokhova et al. 2013), and seasonal changes in copepod carotenoid content have been linked to seasonal shifts in predation pressure relative to UVR exposure (Hansson 2004). Consistent with such a response, in the present study the absolute astaxanthin content in copepods was significantly negatively related to the YOY fish predation, and predation was also among the top variables selected by the MLR models. However, the same was not true for the rate of change, which was unrelated to YOY predation. A plastic adaptation to predation would require the copepods to reduce their carotenoid content in response to chemical signals from the predator (Hylander et al. 2012), which should be reflected in a negative rate of change during periods of increased fish activity. Thus, it seems unlikely that copepods in Lake Simoncouche reduced their carotenoid content primarily in response to predation pressure.

In conclusion, our results together with previous observations elsewhere show that the accumulation of carotenoids is not in all lakes driven by UVR exposure. Instead, we here suggest that astaxanthin may also be used for other purposes than photoprotection, such as for antioxidant protection offered by carotenoids to prevent fatty acid oxidation when accumulating lipid reserves. Given the seasonal correlation of esterified astaxanthin with fatty acids, we also conclude that astaxanthin reserves are accumulated together with fatty acids during periods of high food abundance, and that they are then depleted during egg production, likely due to the transfer of both lipids and carotenoids to the eggs. These dynamics of seasonal reserve accumulation and investment into reproduction do not depend on UVR exposure and may thus affect carotenoid accumulation in any system where overwintering zooplankton experience resource scarcity. However, such dynamics may potentially be overridden by photoprotective functions in highly UVR-exposed systems. The results further point to the need for closer attention to the esterification status of astaxanthin when investigating the ecological roles of this carotenoid.

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