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ORIGINAL ARTICLE

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Saving for the future: Pre-winter uptake of algal lipids supports copepod egg production in spring

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Abstract

1. The freshwater copepod *Leptodiaptomus minutus* in boreal lakes has its main annual reproductive period at the end of winter. This follows months of ice cover and limited food production, yet the females transfer large quantities of algal-derived carotenoids (predominantly astaxanthin) and fatty acids (FAs) to their eggs at this time, thereby providing the offspring with antioxidant protection and energy reserves.
2. We hypothesised that this winter transfer of carotenoid pigments and FAs is based on accumulated reserves that are reinvested into reproduction (i.e. capital breeding). This strategy would allow the animals to produce offspring in time for the nauplii to feed on the spring phytoplankton bloom, thus gaining a competitive advantage.
3. To test this hypothesis, we evaluated the seasonal production of precursor carotenoids and essential FAs by the phytoplankton, the amounts of these compounds required for egg production and the transfer rates from phytoplankton to copepod eggs.
4. Pelagic primary production vastly outweighed the demand for copepod eggs during summer–autumn. However, the major peak of egg production in spring could not be sustained by the low phytoplankton productivity during winter, indicating reliance on previously accumulated reserves as hypothesised.
5. High rates of lipid reserve accumulation in *L. minutus* in late autumn and early winter accounted for up to 128% (astaxanthin precursors) and 70% (FAs) of the daily production by the phytoplankton, further indicating the importance of pre-winter primary production for reserve building in this copepod.
6. During winter, the sum of carotenoid pigments as well as the sum of essential FAs stocked in copepods exceeded the concentrations in the seston. Consequently, adult copepods act as a lipid storage pool linking the biosynthesis of carotenoids and FAs by primary producers in autumn to the production of copepod eggs at the end of winter.

KEYWORDS
antioxidant, astaxanthin, capital breeding, n-3 fatty acids, zooplankton reproduction

1 | INTRODUCTION

One of the key life-history traits that contribute to the ecological success of copepods is their ability to stay active during winter and reproduce immediately before the onset of primary production in spring, giving them an advantage over diapausing species such as many cladocerans and rotifers (Allan, 1976). In order to maintain their physiological activity during the hibernal period of food scarcity,

copepods, like many other animals, may rely on lipid reserves previously accumulated (Maps, Record, & Pershing, 2013; Varpe, 2012). In addition to storage lipids, copepods often have higher concentrations of carotenoids during winter than in summer (García, Pérez, Diéguez, Ferraro, & Zagarese, 2008; Hairston, 1979a; Hansson, 2004), and both classes of compounds may be subsequently transferred to eggs (Schneider, Grosbois, Vincent, & Rautio, 2016).

Reserve building may be seen in the context of "capital breeding," in which the resources required for reproduction are derived from previously accumulated reserves. This contrasts with "income breeding," where concurrent or recent food intake is required to supply the material and cover the energetic costs of reproduction (Stephens, Boyd, McNamara, & Houston, 2009; Varpe, Jørgensen, Tarling, & Fiksen, 2009). Although the concept of capital breeding has placed emphasis on the accumulation of energy reserves (rather than specific essential molecules), it may be extended to certain molecules such as carotenoids and essential fatty acids (FAs) that zooplankton cannot synthesise de novo and thus need to acquire from their diet. For example, copepods are able to convert their major carotenoid, astaxanthin, from phytoplankton pigment precursors such as zeaxanthin, β,β-carotene and potentially lutein (Caramujo, de Carvalho, Silva, & Carman, 2012; Rhodes, 2006). Carotenoids in copepod eggs may not only provide photoprotection to nauplii (Hairston, 1979b) but have also been suggested to stimulate naupliar metabolism via physiological replacement of oxygen allowing for rapid metabolism of energy reserves (Łotocka, Styczynska-Jurewicz, & Błędzki, 2004).

In the case of FAs, the question of essentiality is more complex. All animals including crustaceans require specific combinations of polyunsaturated FAs (PUFAs) not only for growth and reproduction but also for other physiological processes. For example, *Daphnia* specifically retain n-3 FAs, and a high ratio of n-3:n-6 FAs reflects good nutritional conditions (Taipale, Kainz, & Brett, 2015). Additionally, high dietary concentrations of docosahexaenoic acid (DHA; 22:6n-3) promote egg production and growth in zooplankton (Evjemo, Tokle, Vadstein, & Olsen, 2008; Müller-Navarra, Brett, Liston, & Goldman, 2000), while eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6) are important precursors for eicosanoids, a group of signal molecules (Brett & Müller-Navarra, 1997), and stearidonic acid (SDA; 18:4n-3) is associated with increased winter survival (Mariash, Cusson, & Rautio, 2017). Like other animals, crustaceans cannot synthesise short-chain n-3 or n-6 PUFAs such as α-linolenic acid (ALA; 18:3n-3) or linoleic acid (LNA; 18:2n-6) de novo (Parrish, 2009). Many animals, including copepods, can, however, convert ALA and LNA, via elongation and desaturation, to PUFAs such as DHA, EPA or ARA respectively; albeit with limited efficiency (Brett & Müller-Navarra, 1997). This ability varies among copepods and is not known for each species. Therefore, the total pool of n-3 and n-6 PUFAs, respectively, is mainly limited by the availability of each group in the food source. Consequently, lack of appropriate diet during reproduction when certain molecules are physiologically required may increase the success of populations that have acquired suitable reserves prior to the period of scarcity.

In the present study, we investigated how reserve build-up may relate to a capital breeding strategy. Specifically, our goal was to determine whether resource limitation during winter months may force certain copepods to rely on previously accumulated reserves for egg production, and whether such limitation exists for all of the assessed compounds or only for certain types. In order to do this, we studied a bivoltine population of the diaptomid copepod *Leptodiaptomus minutus* that has two main reproduction peaks: a major one in late winter and a smaller second peak at the end of summer. We first estimated the production of astaxanthin precursor carotenoids and n-3 FAs in the seston for one complete year. These groups of compounds were chosen due to their essential role in zooplankton nutrition, that is, they cannot be produced de novo by the copepods and thus need to be accumulated from dietary sources. However, since we do not know the capabilities of *L. minutus* to convert DHA and EPA from shorter chain n-3 precursors, we looked at the sum of n-3 PUFAs as a whole that we considered as essential FAs. We calculated the accumulation and loss rates of these compounds in the adults of *L. minutus*, and by comparing these values to the respective concentration in eggs, we assessed to what extent the above-mentioned sources contribute to copepod reproduction. Additionally, we determined the standing stocks of astaxanthin and n-3 FAs in seston and copepods in different times of the year. This allowed us to evaluate the importance of these biochemical compounds at certain stages of the copepod life cycle, as well as to identify periods of potential limitation for copepod production. We hypothesised that accumulation of astaxanthin and n-3 FAs in this copepod varies according to the seasonal availability of resources and the timing of reproduction, leading to seasonally timed accumulation patterns, build-up of reserves and maternal investment into reproduction. This hypothesis is based on the observation that phytoplankton abundance is generally low from January to March and that copepods most likely do not feed during this period (Rautio, Mariash, & Forsström, 2011) that precedes their annual main reproductive period.

2 | METHODS

2.1 | Study site

Lake Simoncouche is a mesotrophic lake situated in the Laurentides Wildlife Reserve in Quebec, Canada (48.23°N, 71.25°W; elevation 347 m a.s.l.). This dimictic lake covering an area of 87 ha is relatively shallow (Z_{mean} = 2.2 m, Z_{max} = 8 m) and is entirely surrounded by boreal forest. During recent years, the ice cover has typically been forming towards the end of November and has been melting during the second half of April. Following 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 and 8.3 mg C L^{-1} and the photic zone reaches the bottom. However, UV radiation is quickly attenuated (1% penetration depth at 380 nm <1 m). The crustacean zooplankton community of the lake comprises eight copepod species (*L. minutus*, *Epischura lacustris*, *Aglaodiaptomus spatulocrenatus*, *Cyclops scutifer*, *Mesocyclops edax*,

Mesocyclops leuckarti, *Eucyclops speratus* and *Tropocyclops prasinus*) and five cladocerans (*Bosmina* spp., *Daphnia* spp., *Diaphanosoma* spp., *Holopedium glacialis* and *Leptodora kindtii*). Phantom midge larvae (*Chaoborus* sp.) can also be observed occasionally. The community is dominated by *L. minutus* that are present throughout the year forming two distinct cohorts (autumn–winter and a spring–summer), and depending on the season contribute up to 93% (in winter; G. Grosbois, unpublished data) to the total crustacean zooplankton biomass. Potential fish predators on zooplankton include brook trout *Salvelinus fontinalis*, white sucker *Catostomus commersoni* and several species of minnows (Cyprinidae).

2.2 | Sampling of copepods and seston

The abundance of *L. minutus* life stages including eggs was assessed in Lake Simoncouche from 19 May 2011 to 23 May 2012 on a weekly basis during the open-water period and bi-weekly when the lake was ice covered. The quantitative samples (4–20 L) were collected using a 2-L cylindrical water sampler equipped with a messenger-controlled closing mechanism (Limnos Ltd., Turku, Finland) in regular intervals (either 1 or 1.5 m) from the whole water column. When the lake was ice covered, sampling was conducted through a hole. Zooplankton were preserved in formaldehyde (4% final concentration) until counting. Entire samples or aliquots (typically 50% of the total sample volume) were sedimented using Utermöhl chambers and counted on an inverted microscope (Zeiss Axio Observer A1, 50× magnification). Females were identified according to the morphology of their P5 legs (Wilson & Yeatman, 1959). All eggs, either attached to *L. minutus* females or free detached eggs, were counted. On average, about 50 adult *L. minutus* were counted in each sample. During the winter months, the abundance values of adult *L. minutus* varied considerably and suggested an increase of abundance in mid-winter without any preceding stages (C5 or other copepodites) present. Following Cooley (1973), we considered such de novo recruitment implausible and assumed instead that the comparably low numbers detected in the first half of winter did not represent the true abundance, possibly due to some copepods entering a dormant state and staying close to the lake bottom. Instead, we averaged abundance from 22 February to 2 May and set this value as constant abundance from 12 December to 3 April. During each sampling occasion, temperature at 1-m depth intervals was recorded.

Copepods for pigment and FAs analyses were collected as integrated samples from the whole water column (0–6 m) at the deepest point of the lake on 23 occasions from 4 December 2011 to 7 May 2013. Zooplankton was collected 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. 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) to allow time for gut evacuation. The following day, adult *L. minutus* were individually picked from CO_2-sedated zooplankton samples with a pair of forceps. If present, egg sacs were removed from female copepods. On four dates (2 May and 16 May 2012 as well as on 14 May and 13 September 2015), egg samples were obtained by transferring egg-carrying females into a separate dish, then carefully squeezing their abdomen so that they released the egg sacs and subsequently removing the females so that the eggs remained behind. Between 650 and 2,700 eggs per sub-sample were transferred to 1.5-ml plastic tubes. On four dates from 13 June to 20 July 2012, adult *L. minutus* were rare and the net tows were dominated by copepodite CIII to CV stages. Because these stages are considerably smaller than adults, a higher number of individuals was required to obtain a sufficient amount of biomass for carotenoid and FA analyses (typically 0.5 mg dry mass). 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 adult copepods were collected using a pair of forceps. Each sub-sample of adult copepods contained 100–200 individuals, with three sub-samples per analysis. The animals were transferred into 1.5-ml plastic tubes and stored at −80°C until they could be freeze-dried. Dry mass of adult copepods was determined on a microbalance (±2 μg, XP26; Mettler Toledo, Columbus, OH, U.S.A.) from three freeze-dried sub-samples per sampling date, each containing 100–200 individuals. Egg dry mass was estimated on the same microbalance as the average egg mass per weighed freeze-dried egg sample, averaged for all samples. This value (0.13 ± 0.05 μg per egg) was close to the one based on a volumetric estimate (0.15 μg per egg), which was derived from carbon content assuming 0.4 μg C μg^{-1} of dry mass (Huntley & Lopez, 1992).

Water destined for the assessment of seston pigments and FAs was collected on the same dates as copepods at regular depth intervals (either 1 or 1.5 m) including a sub-surface sample using the Limnos water bottle and water samples were pooled for each layer (epilimnion and, when the lake was stratified, metalimnion). 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 sub-samples per analysis of each layer) in the laboratory on the same day. The filtration volume ranged from 0.1 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 aluminium foil and stored in airtight plastic bags at −80°C until freeze-drying, after which they were stored at −50°C.

2.3 | Pigment analysis

Carotenoid and chlorophyll pigments in seston, copepods and eggs were analysed by reversed-phase high-performance liquid chromatography (HPLC). The frozen seston filters were extracted by rod sonication (Microson XL2000; Misonix, Farmingdale, NY, U.S.A.; three times 20 s on ice at 10 W) in 95% (v/v) aqueous methanol. Zooplankton samples were extracted in 90% (v/v) aqueous acetone, homogenised for 2 min (Caframo R2R1 tissue grinder; Wiarton, Ontario, Canada) on ice and then sonicated as described above. This protocol enabled optimal extraction of carotenoids from zooplankton samples (Rautio, Bonilla, & Vincent, 2009). Pigments were extracted for 30 min on ice (seston) or overnight at −20°C (zooplankton) under argon

atmosphere, then centrifuged and filtered through 0.2 μm polytetrafluorethylene membrane filters (VWR International, Mississauga, Ontario, Canada) and stored at 4°C in the dark under argon gas until HPLC analysis within 48 hr. Fifty microlitres were injected into an Accela 600 HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a Hypersil Gold C8 column (150 mm × 4.6 mm, 3 μm particle size; Thermo Scientific) protected by a Hypersil Gold C8 guard column (10 mm × 4 mm, 3 μm particle size; Thermo Scientific) using the HPLC protocol of Zapata, Rodriguez, and Garrido (2000). The run time was 40 min for seston samples and 60 min for zooplankton. Peaks were detected by photodiode array spectroscopy (350–700 nm; slit width: 1 nm) and a fluorescence detector (excitation: 440 nm; emission: 650 nm). Pigments were identified according to retention time and spectra of known standards (see Appendix S1 for details); 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, Villeneuve, & Vincent, 2005; Rautio et al., 2009). Mono- and diesters of astaxanthin were identified according to Snoeijs and Häubner (2014) by separating the first and second clusters of peaks.

2.4 | Fatty acid analysis

Lipids from copepods, egg samples and seston filters were extracted and transmethylated in a one-step reaction in methanol:toluene: acetyl chloride (4,000:1,000:125) at 90°C for 20 min; the resulting fatty acid methyl esters (FAMEs) were separated from non-FAME components by addition of water and hexane (Lepage & Roy, 1984).

FAMEs were analysed by gas chromatography–mass spectrometry (GC-MS) using an Agilent 7890A chromatograph (Agilent Technologies, Santa Clara, CA, 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 with electronic pressure control) and the temperature ramp was as follows: 70°C for 1.5 min followed by an increase of 20°C/min until 110°C, an increase of 12.5°C/min until 160°C and an increase of 2.5°C/min 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 based on 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) versus an internal standard (nonadecanoic acid) using calibration curves based on known standard concentrations (see Appendix S2 for detailed information on the standards used).

2.5 | Primary productivity

The productivity of pelagic algal assemblages was quantified from ^{14}C-bicarbonate uptake in multiple incubations under a gradient of irradiance conditions on five dates in 2012 and on eight occasions in 2014/2015. Integrated water samples containing natural densities of phytoplankton were taken from epilimnion and metalimnion (when present), sieved through a 50-μm net to remove grazers and filled into 20-ml glass scintillation vials. A sample of raw water was kept in an airtight vial at 4°C for subsequent quantification of dissolved inorganic carbon (DIC) via catalytic combustion at high temperature in a TOC-VCPH analyser (Shimadzu, Kyoto, Japan). To each incubation vial, 50 μl of ^{14}C-bicarbonate solution (80 μCi/ml) were added, and after thorough mixing, subsamples of 200 μl were taken for total activity assessment and placed in plastic scintillation vials already containing 200 μl of ethanolamine. The glass vials were incubated for 1–2 hr in six irradiance treatments (0%, 2%, 3%, 7%, 16% and 73% daylight), using a grid of white plastic chambers covered by differing numbers of black mesh layers as in Rae and Vincent (1998) to simulate the photosynthetically active radiation (PAR) gradient with depth. Incident PAR was measured at the surface at 15–30 min intervals during the incubations either using a PUV-2500 profiler radiometer (Biospherical Inc., San Diego, CA, U.S.A.) or a surface PAR-meter connected to a Li1000 data logger (LiCor, Lincoln, NE, U.S.A.). These instruments were also used to obtain the vertical light profile, from which the diffuse vertical attenuation coefficient was calculated as in Schneider et al. (2016). Incubations were placed in shallow water at the shoreline to keep them at lake temperature. After the incubation, the samples were kept in the dark to prevent further photosynthesis. They were filtered onto GF/F glass fibre filters within 2 hr after incubation, and 0.25 ml HCl (0.5 N) was added to remove excess ^{14}C-bicarbonate.

Five millilitres of scintillation cocktail (OptiphaseHisafe; PerkinElmer, Waltham, MA, U.S.A.) was added to the acidified filters. Disintegrations per minute were measured with a PerkinElmer Tri-Carb 2800TR scintillation counter. The pelagic primary productivity (PP, in mg C m^{-3} hr^{-1}) was then calculated as:

$$\mathrm{PP} = \mathrm{dpm}_{\mathrm{sample}} \times \mathrm{DIC} \times \mathrm{vol}_{\mathrm{total\ activity}} \times 1.05/(\mathrm{vol}_{\mathrm{sample}} \times \mathrm{dpm}_{\mathrm{total\ activity}} \times \mathrm{time}) \quad (1)$$

where dpm is counts per minute of either sample of total activity, DIC is the dissolved inorganic carbon concentration (mg C/m^3) in the lake water during the time of the incubation, vol is the volume of the sample (0.02 L) and of the total activity subsamples (0.2×10^{-3} L), respectively, 1.05 is the isotopic discrimination factor correcting for slower uptake of the heavier isotope and time is the incubation time in hours.

PP (average values of two replicate incubations) were fitted by a regression following Platt, Gallegos, and Harrison (1980) to solve the photosynthetic parameters p, α and β:

$$\mathrm{PP} = p(1 - e^{-\alpha E/p})e^{-\beta E/p} \quad (2)$$

where PP (mg C m^{-3} hr^{-1}) is the photosynthesis rate at a given PAR irradiance E (W/m^2).

2.6 | Calculations and data analysis

Several sets of data obtained in 2011–15 were combined to evaluate the annual pattern of copepod abundance and life stages, primary

TABLE 1 Sampling periods for variables used in the calculations

Variable	Sampling period	Sampling occasions
Copepod abundance and life stages	May 2011 to May 2012	42
Carotenoids and fatty acids in copepods and seston	December 2011 to May 2013	22
Astaxanthin in eggs	May 2012, May and September 2015	4
Fatty acids in eggs	May and September 2015	2
Phytoplankton biomass	December 2011 to September 2013, June to September 2014	30
Primary productivity	May to October 2012, May 2014 to February 2015	10

production of pigments and FAs and the occurrence of these compounds in copepods and eggs. Table 1 shows the time period and number of sampling occasions for each variable.

Precursor pigment and FA production in the lake seston were obtained as a function of PP (i.e. production per day) and pigment and FA concentration. In a first step, water-column PAR irradiance was calculated on each sampling date for each hour of the day in each metre of the water column from surface PAR values obtained from a meteorological station at the shore of the study lake (hourly data averaged over 14 days to compensate short-term weather conditions) using the diffuse vertical attenuation coefficient. The attenuation of the ice-cover was taken into account as in Schneider et al. (2016). PP (Equation 2) was then modelled as a function of PAR using the photosynthetic parameters p, α and β for the closest date of PP versus E measurements. When the lake was ice covered, the PP parameters obtained on 4 February 2015 were used, as this was the only PP assessment available in winter. The resulting PP in each metre of depth was multiplied by a depth weighing factor accounting for the bathymetry of the lake, that is, the volume of the respective 1-m layer divided by total lake volume. Thus, values close to the surface contributed more to the lake average than those at the lake bottom, as the surface layer is much more extensive. After this correction, productivity values were summed up within epilimnion and metalimnion according to lake stratification. This factor expressing the relative growth of phytoplankton biomass was multiplied by the concentrations of astaxanthin precursor pigments (sum of lutein, zeaxanthin and β,β-carotene) and the sum of n-3 FAs on that date to obtain a production estimate for precursor pigments and n-3 FAs in the lake seston. The production rates and standing stocks in the epilimnion and metalimnion were combined as a weighted average based on the respective volume of each layer.

The rate of change in copepod content of astaxanthin and n-3 FAs (ng ind^{-1} day^{-1}) was estimated from seasonal changes in copepod content of the respective compounds as in Schneider et al. (2016). In brief, a penalised cubic regression spline was fitted on copepod astaxanthin and n-3 FA concentrations. The rate of change was then calculated as the first derivative of the regression spline. To compare the content and change in copepods with standing stock and productivity in the seston, copepod values were normalised to the unit volume by multiplying them with copepod abundance.

The transfer rates of astaxanthin and FAs from adult copepods to eggs were estimated based on egg production rate on a given date combined with egg-specific contents of astaxanthin and n-3 FAs. These contents were measured on several occasions directly in egg samples during both the winter and the summer reproduction peak (Table 1). Egg development time was estimated based on epilimnetic water temperature according to Cooley and Minns (1978): $D = a\ (T - \alpha)^b$, where D is the development time in hours, T is the water temperature in °C and the constant parameters are $a = 349{,}637$, $\alpha = -9.61$ and $b = -2.51$. Daily egg production was then calculated as $P_{egg} = 24 \cdot \text{egg abundance}/D$. This value was multiplied by the egg-specific carotenoid and FA contents to obtain the transfer rates of these compounds from copepods to eggs.

3 | RESULTS

3.1 | Seasonal cycle of *L. minutus*

The high sampling frequency of copepod seasonal abundance allowed for precise temporal attribution of each life stage (Figure 1). Two cohorts of *L. minutus* could be distinguished within a year, with the first cohort hatching in April–May and the second one hatching in August–September. Beginning with these egg peaks, the succession of developmental stages could be traced for both cohorts during the ice-free period (Figure 1a–d). The first peak of egg abundance in spring appeared at the time of ice-off. During the ice-covered winter period, only adult *L. minutus* were found in the water column as water temperature and chlorophyll *a* concentration reached their annual minima (Figure 1e–f).

Leptodiaptomus minutus eggs were most abundant in April (110 eggs/L), while the second peak in September was much lower (14 eggs/L; Figure 1a). However, egg production rates, as estimated based on temperature-dependent development time, had more similar maximum values in spring (6.3 eggs L^{-1} day^{-1}) and in summer (3.6 eggs L^{-1} day^{-1}). The estimated numbers of total eggs produced by the summer cohort were 146 eggs/L from July to October, while the winter cohort produced 217 eggs/L from March to May and an additional 25 eggs/L in June.

3.2 | Production of astaxanthin precursors and FAs in phytoplankton

The concentrations of the three astaxanthin precursor carotenoids present in the seston (lutein, zeaxanthin and β,β-carotene) were strongly correlated with each other ($r > .9$), and their estimated production did thus follow the same seasonal pattern (Figure 2a). In general, zeaxanthin was the most abundant carotenoid, followed by β,β-carotene and lutein. Astaxanthin precursor productivity (all three pigments summed together) was low when the lake was ice covered

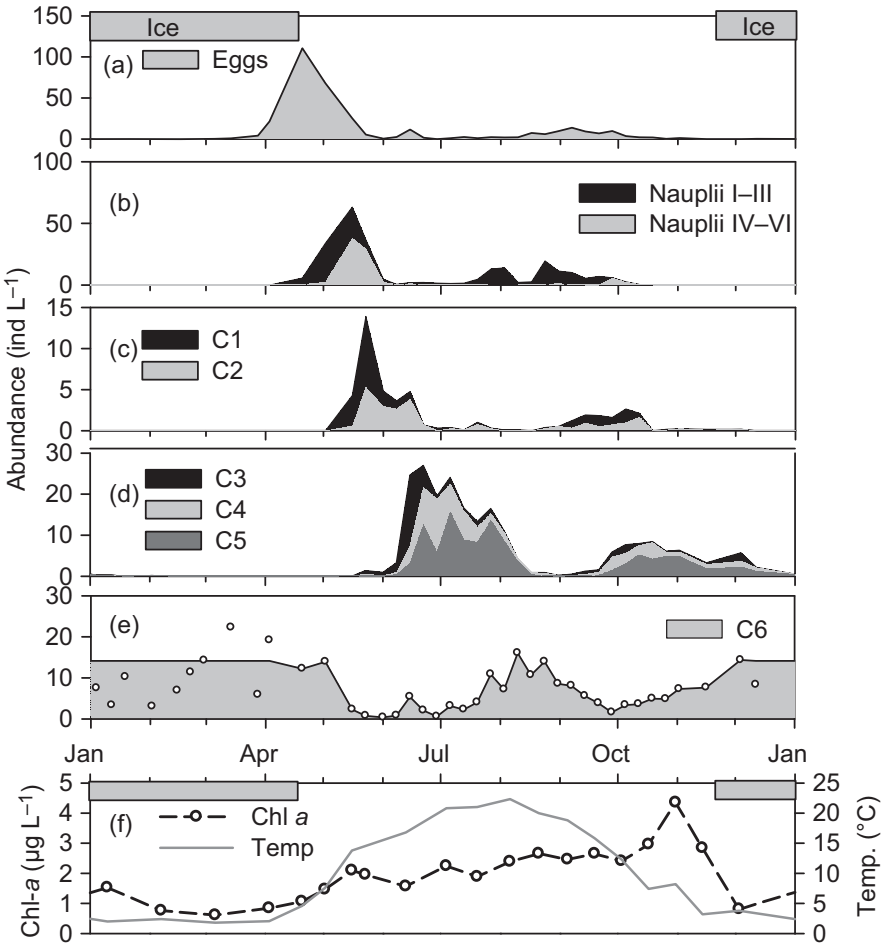

FIGURE 1 Annual population dynamics of *Leptodiaptomus minutus* (a–e) and seasonal changes in ~~chlorophyll~~ *a* (chl *a*) and temperature (f) in Lake Simoncouche. Two cohorts can be distinguished according to the progression of developmental stages (a–e). For the winter period, adult abundance (C6) was smoothed using an average winter abundance value (e)

ranging from 0.0006 µg L^{-1} day^{-1} in January to 0.04 µg L^{-1} day^{-1} in April (Figure 2a). Precursor productivity then increased to its maximum values of 3.4 µg L^{-1} day^{-1} in July–August. During the autumnal mixing period, the productivity of astaxanthin precursors decreased from 0.8 µg L^{-1} day^{-1} in September to 0.06 µg L^{-1} day^{-1} in November. Similar to the astaxanthin precursor carotenoids, seston productivity of the sum of n-3 FAs was low during most of the winter (<0.1 µg L^{-1} day^{-1} from January to March), but was characterised by distinct productivity peaks in early spring and in late autumn (3.9 µg L^{-1} day^{-1} in April and 2 µg L^{-1} day^{-1} in November) and by a comparably less pronounced mid-summer maximum of 14 µg L^{-1} day^{-1} in June (Figure 2a).

3.3 | Carotenoid and FA content in copepod adults and eggs

The rate of change in copepod carotenoid content showed one 3-month period of net accumulation from October to January (positive rate of change, Figure 2b–c) and two periods of net loss (negative rate of change), one from February to April and another one in August–September (Figure 2b). The rate of change in the sum of n-3 FAs showed the same general pattern as carotenoids (Figure 2c). The periods of carotenoid and n-3 FA loss shortly preceded the timing of egg production suggesting that astaxanthin and FAs were transferred to the eggs in the course of reproduction (Schneider et al., 2016). The net uptake rates of both astaxanthin and n-3 FAs reached their maximum in early December: 0.45 ng ind^{-1} day^{-1} of astaxanthin and 4.11 ng ind^{-1} day^{-1} of n-3 FAs. Normalised to the water volume, the maximum uptake rates per individual copepod were 4.3 ng L^{-1} day^{-1} for astaxanthin and 32.6 ng L^{-1} day^{-1} for n-3 FAs (Figure 2b,c).

Phytoplankton productivity of astaxanthin precursors and FAs largely exceeded the demand by adult copepods as determined from net accumulation rates during the open-water period. However, copepod net uptake rates of astaxanthin precursors and of n-3 FAs in January (0.81 and 14.7 ng L^{-1} day^{-1}) accounted for 128% and 70% of the respective phytoplankton productivity of these compounds (0.63 and 21.1 ng L^{-1} day^{-1}), which remained relatively low until March (Figure 2b–c).

Egg carotenoid content varied among years. The two samples in May 2012 contained 0.51 and 0.27 ng per egg of astaxanthin, while in 2015 egg astaxanthin content was only 0.03 (May) and 0.05 (September) ng per egg. This difference resulted from a much lower content of astaxanthin mono- and diesters in eggs, which together accounted for 29%–32% in 2015 as compared to 76%–77% in 2012. Egg total FA content was 3.2 and 9.6 ng per egg in May and 7.5 ng

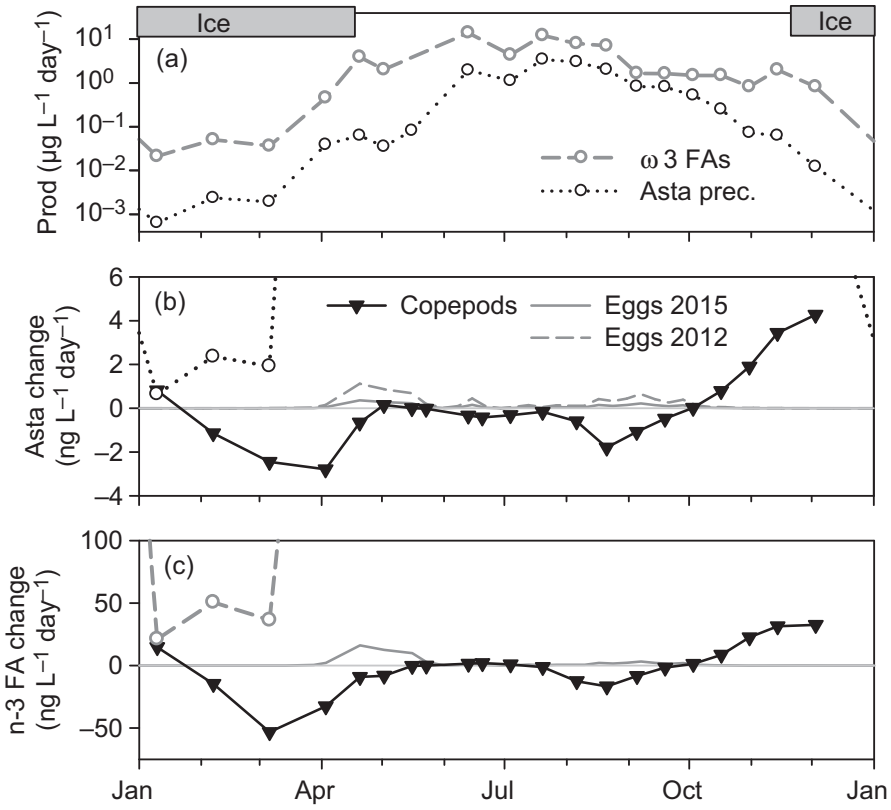

FIGURE 2 Production of astaxanthin precursors and n-3 fatty acids in the lake seston (a), change in copepods and transfer to eggs of astaxanthin (b) and n-3 fatty acids (c). Panel (a) shows the annual pattern of seston production on a logarithmic scale, while very low production values on some winter dates also appear on panels (b) and (c)

per egg in September. The percentage of n-3 FAs was higher in May (48%–51%) than in September (10%). These values were combined with the egg production rate to estimate the transfer of astaxanthin and FAs from the copepods to their eggs. These transfer rates were highest during ice-off at the peak of egg production in April (Figure 2b–c). In the case of astaxanthin, the transfer to eggs at peak reproduction surpassed phytoplankton productivity of astaxanthin precursors during the preceding winter months (Figure 2b). Egg production during the same period amounted to 38 ng astaxanthin L^{-1}, which was 20% of the loss in copepods. In the summer cohort, astaxanthin was reduced by 64 ng/L from July to September corresponding to 26 ng/L, or 40%, astaxanthin investment into eggs. Using the lower egg astaxanthin concentration measured in 2015, the percentages of reinvestment into eggs were 7% in winter and 14% in summer. n-3 FA reduction in copepods was 2.02 µg/L in winter and 0.57 µg/L in summer, corresponding to n-3 FA investment into egg production of 0.56 µg/L (28%) in winter and 0.13 µg/L (23%) in summer respectively. However, since our samples included females and males, the role of egg investment per individual female may have been underestimated (females comprised roughly 40% of the adult population).

3.4 | Distribution of astaxanthin and FA standing stocks in plankton

Copepods served as a major store of carotenoids and FAs in Lake Simoncouche, particularly during winter. The astaxanthin stocked in adult *L. minutus* exceeded seston concentrations of precursor carotenoids throughout the winter months and until early May (up to 0.4 µg/L versus 0.04 µg/L in March; Figure 3a). Similarly, from

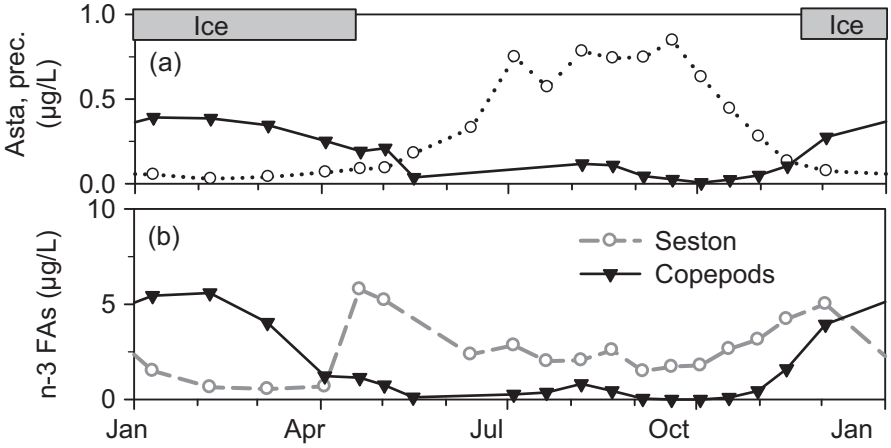

FIGURE 3 Allocation of astaxanthin and its precursors, respectively (a), and n-3 fatty acids (b) in lake seston and copepods

December to April, n-3 FA were more abundant in copepods than in the seston (e.g. n-3 FA in March: 5.6 μg/L in copepods versus 0.54 μg/L in the seston; Figure 3b).

4 | DISCUSSION

Our results provide evidence of capital breeding in the boreal copepod *L. minutus* reproducing under ice with a strong link between investment of astaxanthin and FAs in eggs and the concurrent decline of these lipid reserves in adult copepods. The astaxanthin pigmentation and essential FA content of the copepods changed markedly with season, but did not follow the stocks and production of these compounds in the seston. With the exception of winter, carotenoids and n-3 FAs in the seston diet exceeded the demands by the copepods. The period of most pronounced astaxanthin and FA accumulation in copepods around the onset of winter preceded reproduction by several months, while the loss rates overlapped with the timing of lipid transfer to the eggs. Moreover, despite differential seasonal abundance patterns of astaxanthin precursors relative to n-3 FAs in the lake seston, changes in astaxanthin and n-3 FA content in copepods were correlated. Their concurrence in copepods throughout the year indicates that these changes may be driven by the same controlling factors.

Lipid metabolism plays a crucial role in the life cycle of marine copepods (Kattner et al., 2007). Observations in Antarctic copepods showed that hibernal lipid reserves are primarily invested into reproduction rather than overwintering (Hagen & Schnack-Schiel, 1996). Some polar marine copepods may graze on sedimenting sea-ice algae to support reproduction (Runge, Therriault, Legendre, Ingram, & Demers, 1991), yet, this option appears to be limited to certain marine systems that offer appropriate physical conditions (Hirche & Kosobokova, 2003). Lake ice lacks the brine channels and their associated high concentrations of diatoms that characterise sea ice, and we are not aware of evidence of a similar contribution of ice algae to secondary production in fresh waters, although ice-associated algae are known to occur in frazil ice in rivers (Frenette, Thibeault, Lapierre, & Hamilton, 2008). Under-ice reproduction must therefore be highly dependent on stored reserves. This is consistent with our earlier observations on copepods in Lake Simoncouche that showed pre-winter accumulation of astaxanthin together with reserve lipids and subsequent investment of both astaxanthin and FAs into egg production at the end of winter (Schneider et al., 2016).

The seasonal development of *L. minutus* stages observed here is consistent with observations of this species elsewhere showing reproduction peaks in winter–spring and late summer (Cooley, 1973), and with other records of calanoid copepods reproducing in late winter under the ice (Herzig, Anderson, & Mayhood, 1980; Rautio et al., 2011; Wærvågen & Nilssen, 2010). The small egg peak in June (Figure 1a) may represent a separate cohort (Cooley, 1973), but similar to Cooley's observations this peak dissipated in the following life stages, which made it difficult to follow. It is highly unlikely that it represents a generation on its own, since the development from egg to adult takes about 4 months in spring–summer and 2–3 months in autumn (Figure 1a–e). The comparison with water temperature and chlorophyll *a* concentration suggests that the winter–spring egg peak is timed so that the new cohort of nauplii will reach their feeding stage in time to utilise the increasing productivity of the lake as temperature rises (Figure 1f).

Phytoplankton production rates of astaxanthin precursors and n-3 FAs differed by three and two orders of magnitude, respectively, between summer and winter. From January to March, PP of both FAs and carotenoids was relatively low but then started to increase already before ice-off. Thus, at egg spawning time (April and May) there was no apparent limitation of resources that were required for egg production. However, it must be taken into account that the oocytes need to mature in the female copepods' ovaries before being deposited in the ovisac. Oocyte maturation strongly depends on ambient temperature and generally takes an amount of time comparable to the embryonic development (Caramujo & Boavida, 1999). There is little information on oocycte maturation time in *L. minutus*, but data are available for other diaptomid copepod species. Two alpine diaptomids have oviducal cycle lengths between 35 and 75 days at 4°C (Jersabek & Schabetsberger, 1995). A laboratory study on four North American diaptomid species (Watras, 1983) provides a formula according to which the oviducal cycle would take more than a year below 6°C, effectively halting reproduction. These results suggest that the short period of primary production preceding spawning is insufficient for oocyte maturation to take place.

The costs of reproduction are not limited to oocyte development, and recent evidence suggests that spermatophore production may require considerable resource investment in copepods (Bjaerke et al., 2015; Burris & Dam, 2015). Although the typical astaxanthin content of spermatophores is not known, our recent results suggest that it might be coupled to FA allocation (Schneider et al., 2016). Assuming that spermatophores are placed within a few days before egg spawning, their production might further contribute to the loss of lipid reserves observed in the population during the reproduction period. Therefore, although astaxanthin precursors and n-3 FAs were produced in the seston at about the same rate as they were transferred to eggs at the end of winter, they were likely limiting for the purpose of reproduction. For astaxanthin, the investment into egg production in April surpassed phytoplankton production of precursor carotenoids during January to March, suggesting that PP during winter is insufficient to directly sustain copepod reproduction (i.e. via income breeding). Reserve accumulation (capital breeding) would provide a solution for this problem implying that the required resources (FAs and astaxanthin) were available to the copepods before the onset of spring primary production.

The period from late autumn to early winter (November to January) appears to be crucial for the accumulation of astaxanthin and FA reserves. Astaxanthin and FAs are both transferred to eggs and have closely resembling seasonal dynamics in the copepods (Figure 2b,c), supporting our hypothesis that both types of biochemical compounds are crucial resources for egg production and that their accumulation is directly related to the copepod life cycle. The roles

of different FAs in both energy storage and structural lipids are well established (Arts, 1999; Brett & Müller-Navarra, 1997), while the functions of astaxanthin in eggs and/or nauplii are still subject to debate. Photoprotection is not required in this boreal lake where UVR is attenuated to 1% or less of sub-surface radiation within the first metre (Schneider et al., 2016); thus, metabolic stimulation would seem a more likely function of astaxanthin in the copepod early life stages. Such stimulation could be achieved by physiological replacement of oxygen (Łotocka et al., 2004) or more indirectly by improving the animal's antioxidant capacity (Gorokhova, Lehtiniemi, & Motwani, 2013). Effective antioxidant protection may be particularly important in the non-feeding young nauplii, which rely on the oxidation of stored lipids to produce energy (Łotocka et al., 2004). The predominance of such catabolic processes in some crustacean life stages may result in increased oxidative stress (Fanjul-Moles & Gonsebatt, 2012), thus increasing the requirement for antioxidant defences.

Although maximum egg abundance was much higher in spring than in summer, the numbers of eggs produced by each cohort were in the same order of magnitude. The reduction of both astaxanthin and n-3 FAs during winter closely corresponded to the amount accumulated in the preceding autumn. In the summer cohort, the accumulation cannot be seen in the data because the individual contents of astaxanthin and n-3 FAs do not seem to change when the winter cohort is replaced by the newly developing adults. Nevertheless, the reduction of both types of compounds in August and September strongly overlaps with egg production, emphasising the essential role of astaxanthin and FAs for copepod reproduction.

The magnitude of zooplankton resource accumulation is most apparent at the ecosystem level. During most of the ice-free period, astaxanthin precursor carotenoids and n-3 FAs are more abundant in the lake seston as compared to copepods, but during winter this relationship is reversed and copepods become the major storage of the derived carotenoid astaxanthin and of n-3 FAs (Figure 3). Thus, environmental fluctuations that affect the zooplankton community may have significant impact on the amount of essential resources stored in the lake ecosystem. For instance, earlier ice-off in the wake of climate change would result in a shorter time span where lipid-rich overwintering copepods are present. Combined with the expected reduction in phytoplankton production of n-3 FAs at rising temperatures (Hixson & Arts, 2016), shifts in the phytoplankton community in late fall/early winter might result in reduced availability of high-quality lipids for aquatic secondary consumers such as young-of-the-year fish. Identifying such key periods for reserve accumulation in zooplankton may thus improve our ability to predict year to year changes in fish recruitment.

In conclusion, these data imply that resource limitation is a likely factor favouring capital breeding in the strongly seasonal, boreal lake environment. During late autumn and early winter, the copepods directly consume large quantities of photosynthetic biomass to build up their reserves of critical molecules for the winter. These resource stocks then enable a reproductive effort that could not be sustained by winter primary production alone.

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