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## LIST OF ABBREVIATIONS

ARA	arachidonic acid (20:4 $\omega$ 6)
Asta	astaxanthin, a carotenoid
Asta <sub>tot</sub>	total astaxanthin concentration in <i>L. minutus</i>
CDOM	colored dissolved organic matter
DHA	docosahexaenoic acid (22:6 $\omega$ 3)
DIC	dissolved inorganic carbon
DOC	dissolved organic carbon
$E_d$	down-welling irradiance
EPA	eicosapentaenoic acid (20:5 $\omega$ 3)
FA	fatty acid
FAME	fatty acid methyl ester
FA <sub>tot</sub>	total fatty acid concentration in <i>L. minutus</i>
GC	gas chromatograph
HPLC	high-performance liquid chromatography
$K_d$	diffuse downwelling attenuation coefficient
$K_{ice}$	attenuation coefficient of ice and snow cover
MAA	mycosporine-like amino acid, a type of PPC
MLR	multiple linear regression
PAR	photosynthetically active radiation
$PP$	primary productivity
PPC	photoprotective compound
PUFA	poly-unsaturated fatty acid, <i>i.e.</i> , a FA with more than one double bonds in its carbon chain
ROS	reactive oxygen species
UVR	ultraviolet radiation



YOY	young-of-the-year, referring to juvenile fish less than one year of age
Z	depth
$\omega$ 3 FA	Omega-3 fatty acid, <i>i.e.</i> , a PUFA with a carbon double bond at the third position from the terminal carbon atom

## SUMMARY

Despite the long period of ice-cover in boreal aquatic ecosystems, knowledge on their winter ecology is limited. One open question is why some copepods show high concentrations of the supposed photo-protective compounds, carotenoids, in absence of light. Carotenoid pigmentation in copepods has been described as a plastic adaptation to the opposing threats of exposure to ultraviolet radiation (UVR) and visual predation. The major carotenoid in copepods, astaxanthin, is a powerful antioxidant that is often esterified to fatty acids suggesting a potential physiological connection to the lipid storage. Copepods may pass lipids and carotenoids on to their eggs to provide early larval stages with energy and antioxidant protection. Carotenoids must be accumulated from primary producers (phytoplankton) and thus, the availability of appropriate diet may limit (or enhance) copepod pigmentation.

This doctoral project aimed at assessing the seasonal pattern of copepod pigmentation and its controlling factors. We followed the pigment and fatty acid content of *Leptodiaptomus minutus* in a Boreal Shield lake for eighteen months covering two winter seasons along with a range of environmental variables representing the major hypotheses regarding carotenoid accumulation in copepods. Potential explanatory variables that were tested included UVR exposure, water temperature, food abundance and quality, predation pressure and copepod egg production. In a follow-up study we estimated the pelagic primary production of carotenoids and fatty acids to quantify the transfer rates of these compounds from seston to copepod to eggs, allowing us to identify potential periods of limitation for copepod reproduction. These observations were complemented by laboratory experiments exposing live copepods to natural levels of UVR, visible light, or darkness at two different temperatures.

Astaxanthin was the predominant carotenoid in copepods and occurred in its 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 changes of copepod total astaxanthin were characterized by net accumulation in late fall of up to  $0.034 \mu\text{g (mg dry mass)}^{-1} \text{d}^{-1}$  followed by a mid-

winter maximum of  $3.89 \pm 0.31 \mu\text{g mg}^{-1}$ . The two periods of net loss ( $-0.018$  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. Experimental UVR exposure did not affect copepod carotenoid content. However, both survival and carotenoid content in copepods were negatively affected by temperature in the warmer treatment.

These results imply that seasonal changes of astaxanthin pigmentation in copepods are strongly related to lipid metabolism rather than to photoprotection. Accumulation and loss of both astaxanthin and fatty acid reserves are related to food availability and copepod reproduction, with potential additional effects of water temperature and predation pressure.

When estimating production and transfer rates of carotenoids and fatty acids on the ecosystem level we found that pelagic primary production vastly outweighed the amount required for copepod egg production during summer, but that the major peak of egg production in spring could not be sustained by the low phytoplankton productivity during winter. High rates of reserve accumulation in *L. minutus* in late fall and early winter corresponded to a considerable portion of the daily production by the phytoplankton emphasizing the critical role pre-winter primary production may play in the copepod life cycle. During winter, astaxanthin and fatty acids stocked in copepods exceeded the respective precursor concentrations in the lake seston, underlining the substantial extent of reserve accumulation on the ecosystem level. Consequently, adult copepods function like a storage bridging the gap between the biosynthesis of carotenoids and fatty acids by primary producers in fall and the production of copepod eggs during late winter.

This thesis is the first to convincingly link copepod carotenoid accumulation to the build-up of lipid reserves, explaining the seasonal dynamics of astaxanthin by reproductive requirements and dietary constraints. Furthermore the results are in line with an emerging base of evidence from a diverse range of systems suggesting that photoprotection is not always the primary function of carotenoids in copepods. Finally our observations underline the central role that zooplankton play for the storage of carotenoids and fatty acids during winter allowing for continued utilization of these important molecules in the lake food web.

## RÉSUMÉ

Malgré une longue période de couverture de glace des écosystèmes aquatiques boréaux, les connaissances sur leur écologie hivernale sont limitées. Une des questions actuellement posées est : « pourquoi certains copépodes contiennent-ils des concentrations élevées de caroténoïdes, agents supposés photo-protectifs, en absence de lumière? » La pigmentation en caroténoïdes des copépodes a été décrite comme une adaptation plastique aux menaces antagonistes de l'exposition au rayonnement ultraviolet (RUV) et de la prédation visuelle. Le principal caroténoïde des copépodes, l'astaxanthine, est un puissant antioxydant qui est souvent estérifié à des acides gras suggérant une potentielle connexion physiologique avec les réserves lipidiques. Les copépodes peuvent transférer les lipides et les caroténoïdes à leurs œufs afin de fournir l'énergie et la protection antioxydante nécessaires aux premiers stades larvaires. Le zooplancton devant accumuler leurs caroténoïdes du phytoplancton, élément essentiel de leur régime alimentaire, la disponibilité en nourriture peut augmenter ou limiter la pigmentation des copépodes.

Ce projet doctoral a pour objectif d'évaluer le patron saisonnier de la pigmentation des copépodes et d'identifier les facteurs qui le contrôlent. Nous avons suivi le contenu en pigments et en acides gras de *Leptodiatomus minutus* dans un lac du Bouclier canadien pendant dix-huit mois couvrant deux saisons hivernales. L'ensemble des variables environnementales potentiellement influentes suivant les principales hypothèses d'accumulation de caroténoïdes par les copépodes ont aussi été suivies, incluant l'exposition au RUV, la température de l'eau, l'abondance et la qualité de la nourriture, la pression de prédation et la production d'œufs des copépodes. Lors d'une seconde étude, nous avons estimé la production primaire pélagique des caroténoïdes et des acides gras afin de quantifier les taux de transfert de ces molécules du seston vers les copépodes et par la suite aux œufs dans le but d'identifier les périodes potentiellement limitantes pour la reproduction des copépodes. À ces observations se sont ajoutées des expériences en laboratoire exposant des copépodes vivants à des niveaux naturels de RUV, de lumière visible ou d'obscurité, et ce à deux températures différentes.

L'astaxanthine était présente en forme libre aussi bien qu'estérifiée avec un ou deux acides gras. Les esters, qui constituaient entre 62 % et 93 % de l'astaxanthine totale, variaient de façon saisonnière en forte corrélation avec les acides gras. Le changement saisonnier de l'astaxanthine totale des copépodes était caractérisé par une accumulation nette en fin d'automne, jusqu'à  $0.034 \mu\text{g} (\text{mg poids sec})^{-1} \text{j}^{-1}$ , suivie par un maximum hivernal de  $3.89 \pm 0.31 \mu\text{g mg}^{-1}$ . Les deux périodes de réduction nette ( $-0.018$  et  $-0.021 \mu\text{g mg}^{-1} \text{j}^{-1}$ ) coïncidaient avec les pics de production d'œufs au printemps et en été, menant à un contenu minimal en astaxanthine ( $0.86 \pm 0.03 \mu\text{g mg}^{-1}$ ) en automne. Cette période a aussi été caractérisée par la pression de prédation par les jeunes poissons la plus élevée de l'année. L'exposition expérimentale au RUV n'a pas affecté le contenu en caroténoïdes des copépodes. Par contre, la survie des copépodes ainsi que le contenu en caroténoïdes ont été négativement affectés par la température dans les traitements les plus chauds.

Ces résultats impliquent que les changements saisonniers de la pigmentation en astaxanthine des copépodes sont fortement liés au métabolisme lipidique plutôt qu'à la protection contre le RUV. L'accumulation et la perte des réserves d'astaxanthine et d'acides gras semblent liées à la disponibilité de nourriture et à la reproduction, avec de potentiels effets additionnels de la température de l'eau et de la pression de prédation.

En estimant les taux de production et de transfert de caroténoïdes et d'acides gras au niveau de l'écosystème, nous avons trouvé que la production primaire pélagique outrepassait largement la quantité requise pour la production d'œufs de copépodes en été. Par contre, le pic majeur de reproduction au printemps ne pouvait pas être soutenu par la faible productivité du phytoplancton pendant l'hiver. Les taux élevés d'accumulation de réserves par *L. minutus* en fin d'automne et en début d'hiver correspondaient à une partie considérable de la production quotidienne effectuée par le phytoplancton, soulignant le rôle crucial que la production primaire pré-hivernale peut jouer dans le cycle de vie des copépodes. Pendant l'hiver, les stocks en astaxanthine et en acides gras des copépodes dépassaient les concentrations des précurseurs correspondants au niveau du seston du lac, soulignant l'importance de l'accumulation de réserves à l'échelle de l'écosystème. En conséquence, les copépodes adultes fonctionnent comme un entrepôt qui lie la biosynthèse de caroténoïdes et d'acides gras par les producteurs primaires en automne à la production d'œufs de copépodes au printemps.

Cette thèse est la première qui établit un lien convaincant entre l'accumulation de caroténoïdes des copépodes et leurs réserves lipidiques, en expliquant les dynamiques saisonnières de l'astaxanthine par les besoins reproductifs et les contraintes nutritives. Par ailleurs, les résultats s'inscrivent dans une littérature émergente provenant d'une variété d'écosystèmes différents qui suggère que la photo-protection n'est pas toujours la fonction principale des caroténoïdes chez les copépodes. Finalement, nos observations soulignent le rôle central du zooplancton comme entrepôt de caroténoïdes et d'acides gras pendant l'hiver, ce qui permet l'utilisation continue de ces molécules dans le réseau trophique du lac.

Mots clés : copépodes, rayonnement ultraviolet, limnologie hivernale, astaxanthine, caroténoïdes, acides gras, réserves lipidiques

## AUTHOR'S CONTRIBUTION

I made significant contributions to the planning, data collection and execution of all three studies presented in this thesis. The specific contributions in each chapter were as follows (Tobias Schneider = TS).

Chapters I and II: TS, Milla Rautio (MR) and Warwick F. Vincent (WFV) conceived the study design. TS and Guillaume Grosbois (GG) carried out field and lab work. TS analyzed the data and wrote the first draft, which was then finalized together with the co-authors.

Chapter III: TS and MR designed the experiment. TS carried out field and lab work, analyzed the data and wrote the first draft, which was then finalized together with MR.

## INTRODUCTION

### **Problem statement**

Lakes in the boreal biome are typically ice-covered for a substantial part of the year. Nevertheless, they often host an abundant pelagic fauna that stays active during winter (Rigler et al. 1974; Vanderploeg et al. 1992). During the winter season, crustacean zooplankton are confronted with cold temperatures and limited food availability, and they display a variety of overwintering strategies ranging from continued feeding on lower-quality diet to diapausing as permanent eggs in the sediment (Santer 1998; Larsson and Wathne 2006). Some marine copepods are able to survive several months without feeding, metabolizing storage lipids accumulated during the ice-free period (Kattner et al. 2007). The energy is stored in lipid droplets, often visibly colored due to associated carotenoid pigments (Arts 1999; van Der Veen 2005).

Since zooplankton cannot synthesize carotenoids *de novo*, these pigments are ultimately derived from primary producers and often modified by the animals to obtain astaxanthin, the primary carotenoid in crustaceans (Andersson et al. 2003). Astaxanthin is a powerful antioxidant that can be present in free form as well as esterified with fatty acids or bound to proteins (Cheesman et al. 1967; Matsuno 2001; McNulty et al. 2007). Due to their antioxidant capabilities, carotenoids may mitigate oxidative damage that occurs during the exposure to ultraviolet radiation (UVR) (Krinsky 1979; Cockell and Knowland 1999). In spatial comparisons of lakes with different transparency and/or depth, carotenoid content in copepods has been shown to be positively linked to the level of UVR, indicating a photoprotective function of carotenoids (Hylander et al. 2009; Sommaruga 2010). However, since visibly



pigmented individuals are more susceptible to visual predators such as fish larvae, zooplankton may reduce their level of pigment content in presence of predator cues (Hairston 1979a; Hylander et al. 2009).

The occurrence of strongly pigmented copepods in winter under the ice has been documented in several studies (Hairston 1979a; García et al. 2008). These observations suggest that protection from UVR is not always directly driving copepod pigmentation, as solar irradiance is low during winter and is further attenuated by the ice and snow cover. The observation is also interesting because primary production is strongly reduced at that time of the year, limiting the potential uptake of dietary precursors by zooplankton. Copepods that reproduce at the end of winter may require a winter carotenoid reserve to pass part of it on to their eggs, thus supporting the offspring's first life stages (Hairston 1979b). Some proposed functions of carotenoids in nauplius larvae include photoprotection, the use as energy reserves, or metabolic stimulation via their antioxidant properties (Łotocka et al. 2004). Similarly, copepod egg production and hatching success benefits from the availability of other lipids such as essential fatty acids (Broglio et al. 2003; Kattner et al. 2007). As some copepods are known to reproduce during winter when their habitat is ice-covered and pelagic primary production is low (Rautio et al. 2011), they might face a challenge in providing the carotenoids and fatty acids to be transferred to their eggs.

Knowledge on the environmental seasonality in boreal systems appears crucial for the understanding of their ecology. However, studies covering seasonal variation are still rare, and the winter has been seen as a dormant period with only marginal biological activity (Hampton et al. 2015). Revealing the strategies that allow plankton to survive a time with very limited food supply and challenging physical conditions is thus an important objective to provide better understanding of these ecosystems. The aim of this study was to investigate the ecological significance of carotenoid accumulation in crustacean zooplankton in the context of seasonally varying factors such as

temperature, UVR exposure, diet availability and predation, as well as copepod lipid storage and reproduction.

## **Current state of knowledge**

### *Solar ultraviolet radiation and dissolved organic matter*

Ultraviolet radiation designates short-wave electromagnetic radiation adjacent to the visible spectrum and can be artificially divided into three major wavebands, i.e., UV-C (200–280 nm;), UV-B (280–320 nm), and UV-A (320–400 nm) (Cockell and Knowland 1999). UV-C is completely absorbed in the stratosphere and does not reach the Earth's surface. Of the remaining UVR, the highly energetic shorter wavelengths have a higher capability of causing biological damage. Such negative effects include DNA damage, inhibition of photosynthesis, production of ROS, and other harmful physiological responses (Cockell and Knowland 1999; Rautio and Tartarotti 2010). UVR-induced damage occurs either directly by altering molecular configuration (e.g., thymine dimerization in DNA) or via the photochemical generation of reactive oxygen species (ROS), which in turn damage lipids, proteins, and DNA (Rautio and Tartarotti 2010; Burritt and Lamare 2016). In the water column, the attenuation of UVR depends mainly on the concentration of dissolved organic carbon (DOC) and the absorptivity of its chromophoric fraction (CDOM, colored dissolved organic matter) (Morris et al. 1995; Sommaruga 2001).

Aquatic organisms need to balance their life strategies to minimize at the same time hazardous effects of UVR (tissue damage, reduced fertility, death) and the cost of photoprotection (e.g., reduced photosynthesis and slower growth when staying in a depth refuge; elevated susceptibility to visual predators when accumulating pigments). Strategies to minimize the negative effects of UV radiation have evolved on three levels (Cockell and Knowland 1999; Sommaruga 2010): avoidance of UVR

exposure, limiting effects of UVR through photoprotective compounds, and repair of UVR-induced damage by cellular repair mechanisms.

To avoid UV exposure, zooplankton such as *Daphnia* (Rhode et al. 2001) and some copepods (Alonso et al. 2004) show radiation-dependent vertical migration behavior on a diel basis, thus using the water column and dissolved substances as a sunscreen (Cockell and Knowland 1999). In habitats with low DOC concentration or shallow lakes with important wind-driven mixing, zooplankton might not be able to avoid hazardous radiation intensities, and thus rely on the accumulation of photoprotective compounds (PPCs) (Hylander et al. 2009; Rautio and Tartarotti 2010). Some of these compounds such as melanin and mycosporine-like amino acids (MAAs) directly screen UV radiation, while carotenoids protect the cell indirectly by quenching reactive oxygen species (Cockell and Knowland 1999).

Future tendencies of UVR exposure in aquatic systems are subject a variety of counteracting effects (Häder et al. 2015). It is now clear that the Antarctic ozone layer has been recovering since the turn of the century (Solomon et al. 2016). As a result of climate change, CDOM concentrations in inland waters are expected to increase, causing stronger attenuation of solar UVR in the water column (Häder et al. 2015). These reductions in underwater UV irradiance are counteracted by shorter period of ice cover leading to earlier exposure to high UVR levels in spring (Häder et al. 2015). Thus, in temperate lakes the period of UVR exposure is likely to increase while at the same time the dose rate decreases.

#### *Carotenoids and fatty acids in freshwater pelagic crustaceans*

Carotenoids are a class of isoprenoid lipids typically consisting of 40 carbon atoms with alternating double bonds. These lipid-soluble pigments are synthesized by primary producers and are often accumulated at higher trophic levels (Andersson et

al. 2003). Copepods may transfer carotenoids to their eggs together with other components such as lipid and protein reserves (Hairston 1979b; Łotocka et al. 2004).

The major carotenoid in copepods is astaxanthin (Andersson et al. 2003; Łotocka et al. 2004). This carotenoid is only present in some phytoplankton species and thus in most systems has to be converted from precursor carotenoids such as  $\beta,\beta$ -carotene, zeaxanthin and potentially lutein (Matsuno 2001; Andersson et al. 2003; Rhodes 2006). In copepods, a large portion of the total astaxanthin may form mono- or diesters with one or two fatty acids, respectively (Matsuno 2001; Sommer et al. 2006). The ratio of free versus esterified astaxanthin can vary significantly between developmental stages (Łotocka et al. 2004). Astaxanthin esters have been suggested to be accumulated within the lipid storage to counteract lipid peroxidation and thus prevent degradation of fatty acids (Sommer et al. 2006). Carotenoids may also protect copepods against oxidative stress produced during an immune response (van Der Veen 2005).

Hairston (1976) found that carotenoids increase survival rates of copepods exposed to visible (blue) light, the main absorbance region of carotenoids (typically between 400 and 550 nm). UV screening capabilities of carotenoids are relatively weak, but they facilitate the quenching of highly reactive oxygen species generated during UVR exposure. Solar ultraviolet radiation (UVR) is an important source of oxidative stress, and carotenoids have been attributed an indirect photoprotective function (Hairston 1976; Hansson and Hylander 2009; Rautio and Tartarotti 2010) via the quenching of ROS such as singlet oxygen (Kobayashi and Sakamoto 1999). Spatial differences in copepod astaxanthin content among otherwise similar lakes have been successfully explained by the exposure to UVR in the respective lakes (Sommaruga 2010). Water temperature has been proposed as an additional factor controlling carotenoid accumulation in copepods, with the argument of providing additional photoprotection

when cellular repair systems are ineffective due to cold temperatures (García et al. 2008).

Carotenoids make organisms look red, orange, or blue (when bound to proteins; Cheesman et al. 1967). Therefore, they may alter predation pressure by increasing an animal's visibility or providing camouflage (Łotocka et al. 2004). The influence of predation threat and its cross relations with UV threat on carotenoid accumulation in calanoid and cyclopoid copepods have been assessed by several authors. In these studies, predators were either fish (Hansson 2004; Hylander et al. 2009) or salamanders combined with invertebrates (Hairston 1979a). The higher the perceived predation threat was in relation to UVR exposure, the lower was the carotenoid concentration in copepods, and vice versa. These authors suggested that the animals adjust their pigmentation to the proportion of both threats, thus optimizing their overall threat resistance. The expression of carotenoids can be induced by UVR exposure or alternatively be inhibited by predator cues (Hylander et al. 2013; Brüsin et al. 2016).

Fatty acids (FAs) are aliphatic hydrocarbon chains with a terminal carboxyl group that can either be saturated (maximum amount of hydrogen atoms bound to the carbon chain) or unsaturated (some hydrogen atoms are replaced by carbon-carbon double bonds). Typically, FAs occur as part of a larger lipid molecule that consists of a "head" with two or three FA "tails" attached to it.

Lipids can be divided into two general classes: neutral lipids, and polar lipids. Neutral lipids such as triacylglycerols and wax esters are stored to be catabolized when needed, whereas polar lipids such as phospholipids are major constituents of cell membranes (Olsen 1999). Due to their high energy density, lipids are the preferred storage molecules in zooplankton, comprising up to 65% of their dry weight and an even larger proportion of the digestible biomass (Arts 1999). Among pelagic

freshwater crustaceans, the highest total lipid concentrations are found in calanoid copepods followed by cyclopoids and cladocerans (Syväranta and Rautio 2010). Lipids play an important role in overwintering strategies as well as reproduction; lipid content and composition is thus also a function of the life cycle of a given zooplankton, as lipids are passed-on to offspring and provide energy reserves during winter dormancy (Kattner et al. 2007). They constitute the primary energy reserve in marine and freshwater zooplankton, and represent the most variable component of zooplankton total lipid content (Arts 1999). Total lipid content is generally higher and more variable in high-latitude waters than in boreal systems (Syväranta and Rautio 2010). Storage lipids in cladocerans and copepods are often associated with pigments and visible as yellow to orange lipid droplets (Arts 1999). Phospholipids fulfill a crucial structural function as major constituents of all organisms' cell membranes. In aquatic poikilothermic animals (such as zooplankton and fish) living in cold environments, membrane lipids are usually characterized by a high fraction of polyunsaturated FAs (PUFA) (Olsen 1999).

PUFA are defined as FAs with more than one double-bond (Brett and Müller-Navarra 1997). They are usually described by the position of the last double bond as counted from the terminal carbon atom, *e.g.*,  $\omega 3$  and  $\omega 6$  FAs. Important PUFA in zooplankton are arachidonic acid (20:4 $\omega 6$ , ARA), eicosapentaenoic acid (20:5 $\omega 3$ , EPA) and docosahexaenoic acid (22:6 $\omega 3$ , DHA). Many PUFA are regarded essential in animals. Rather than being used as energy reserves they fulfill important physiological functions. For example, PUFA availability in the diet positively influences zooplankton productivity as they are required for egg production (Brett and Müller-Navarra 1997). A central function of PUFA is as cell membrane constituents allowing the organism to maintain membrane fluidity at low ambient temperature (Schlechtriem et al. 2006). Furthermore, certain PUFA are required as precursor substances for various animal hormones (Brett and Müller-Navarra 1997; Kattner et al. 2007). The ratio of specific PUFA differs among taxonomic groups: cladocerans

rely mainly on ARA and EPA, but contain only marginal amounts of DHA, whereas copepods have more DHA than EPA (Persson and Vrede 2006; Brett et al. 2009a; Mariash et al. 2011). The high DHA content in copepods has been linked to their more developed nervous system (Brett et al. 2009b). Since secondary producers have to accumulate essential PUFA from their diet, zooplankton FA composition reflects, among other factors, the FAs available in their food (Kattner et al. 2007; Brett et al. 2009b) and the taxonomic position (Persson and Vrede 2006). In *Daphnia*, temperature and reproductive state affect FA composition (Schlechtriem et al. 2006; Brett et al. 2009b). Potential future changes in water temperature and UVR exposure might affect zooplankton lipid composition indirectly, by modifying algal growth and nutritional quality (Arts 1999; Hixson and Arts 2016).

#### *Seasonal constraints on zooplankton in freshwater systems*

A common feature of lakes situated in the boreal zone is the pronounced seasonality these ecosystems undergo in physical and biotic aspects. Planktonic organisms are challenged by water temperatures close to zero, an ice cover lasting several months, and lack of sunlight. For zooplankton, the winter is characterized by drastically reduced availability of high-quality food due to limited or absent primary production (Sommer et al. 1986).

One of the key life-history traits that contributes 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). Cladocerans either produce resting eggs, or stay active feeding on reduced diet during winter (Larsson and Wathne 2006; Rellstab and Spaak 2009). Similar to *Daphnia*, copepods may show dormancy (*e.g.*, resting eggs or reduced metabolic activity; Dahms 1995) or assimilate benthic carbon channeled to them via the microbial loop (Karlsson and

Sävström 2009). Some copepods reproduce during winter (Rigler et al. 1974), sometimes without additional food intake (Kattner et al. 2007; Rautio et al. 2011). In order to maintain their physiological activity during the hibernal period of food scarcity, copepods, like many other animals, may rely on lipid reserves accumulated ahead of time (Varpe 2012; Maps et al. 2013).

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 pay for the material and energetic costs of reproduction (Stephens et al. 2009; Varpe et al. 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 also cover certain molecules such as carotenoids and essential fatty acids that zooplankton cannot synthesize *de novo* and thus need to acquire from their diet. Consequently, lack of appropriate diet during late-winter reproduction when those molecules are physiologically needed may favor individuals that have acquired reserves prior to the period of scarcity.

### **Objectives and hypotheses**

The overall aim of this thesis is to understand the seasonality, functions, and driving factors of carotenoid accumulation in copepods. By assessing the driver(s), conclusions about the function(s) may be drawn. The work can be split into the following objectives, some of which are covered in more than one chapter (referred to by their Roman numerals).

1. Assess the seasonal change of carotenoid pigmentation in the copepods (I, II).



2. Identify the driving factors (I, III). Based on the state of knowledge as portrayed above, the following hypothesized factors were evaluated as potential drivers of carotenoid accumulation or loss in copepods:
  - a. UVR exposure,
  - b. diet availability (presence of precursor pigments in the diet),
  - c. lipid reserves (correlation with total lipids or a group of fatty acids),
  - d. life cycle (*i.e.*, passing on to offspring, preparation for the spring ice break-up),
  - e. selective predation pressure on pigmented animals.
3. Quantify the transfer of precursor compounds from phytoplankton via copepods to copepod eggs. Based on food availability and reproductive effort, we aimed at identifying critical periods and potential dietary limitations for carotenoid accumulation in copepods (II). We hypothesized that the copepod life cycle is adapted to meet dietary constraints and maximize reproductive output via means of reserve accumulation and capital breeding.

### **Methodological approach and study site**

This work aimed at assessing all of the major hypotheses that have been proposed as candidates to explain carotenoid accumulation in copepods. The approach we chose was to closely follow the seasonal dynamics in one system to obtain a thorough understanding of the processes and interactions that may affect our variable of interest, the carotenoid content in copepods. The diverse range of potential predictor variables would provide the empirical basis on which the different hypotheses could be evaluated. We were able to obtain reliable data on all of the relevant variables allowing us to directly test these hypotheses in an actual ecosystem.

Two important methodological elements of this research project have been defined in the very beginning. The first one was to acknowledge the importance of taking into

account ecological processes during the ice-covered period, which for a long time has been a neglected aspect of limnology (Hampton et al. 2015). This approach was logistically facilitated by the presence of a permanent field station at the lake shore equipped with gear, boats and snowmobiles for both open-water and under-ice sampling. Secondly, the decision to analyze both carotenoids and fatty acids at the high level of precision offered by high-performance liquid chromatography (HPLC) and gas chromatography, respectively. The combination of these approaches led us to one of the major discoveries of this thesis, the co-accumulation of carotenoids and lipid reserves during winter.

For Chapter I, year-round samples and data have been collected in Lake Simoncouche in regular sampling intervals (although sampling frequency was higher during the open-water period) throughout 18 months covering two winter periods. In addition to the absolute content of astaxanthin, we calculated the rate of change based on a penalized smoothing spline fitted on the seasonal data of astaxanthin content. This variable allowed for testing the effects of potential predictors that would influence accumulation and reduction rather than the absolute concentration, such as diet availability or transfer to eggs. The relative importance of different explanatory variables was assessed using an information-based approach combined with multiple regression analysis (Burnham and Anderson 2002). Some of these data were then combined with data on population dynamics collected by my colleague Guillaume Grosbois as well as with measurements of primary productivity to quantify the transfer rates and stocks of both carotenoids and selected fatty acids on the ecosystem level (Chapter II).

Two experiments were performed on live copepods, manipulating exposure to UVR and water temperature (Chapter III). Copepods of the calanoid species *Leptodiaptomus minutus* have been taken from Lac Simoncouche in central Québec.

Cultured algae of the genus *Scenedesmus* were used as diet. The experiments lasted 21 and 18 days.

All of the data presented in this thesis are based on the same population of the copepod *Leptodiptomus minutus* from Lake Simoncouche. This dimictic, mesotrophic lake situated in the Laurentides Wildlife Reserve in Quebec, Canada (48.23 °N, 71.25 °W; 347 m above sea level) and covering an area of 87 ha is relatively shallow ( $Z_{\max} = 8$  m) and is entirely surrounded by boreal forest. It has several inflows and one outflow. 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 and 8.3 mg C L<sup>-1</sup>. The crustacean zooplankton community of the lake consists of six copepod species (*Leptodiptomus minutus*, *Epischura lacustris*, *Aglaodiaptomus spatulocrenatus*, *Cyclops scutifer*, *Mesocyclops edax*, *Tropocyclops prasinus*) and five cladocerans (*Bosmina* spp., *Daphnia* spp., *Diaphanosoma* spp., *Holopedium gibberum*, *Leptodora kindtii*). *Chaoborus* sp. can also 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. The lake harbors 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*.

### **Structure of this thesis**

This thesis is based on the following original research articles, which will be referred to in the text by their Roman numerals I to III. Chapter I has been published in

Limnology and Oceanography, Chapter II is currently in revision, and Chapter III is a manuscript ready for submission.

**I.** Schneider, T., G. Grosbois, W. F. Vincent, and M. Rautio. 2016. Carotenoid accumulation in copepods is related to lipid metabolism and reproduction rather than to UV-protection. *Limnol. Oceanogr.* **61**: 1201–1213, doi: 10.1002/lno.10283. Open access at <http://onlinelibrary.wiley.com/doi/10.1002/lno.10283/full>

**II.** Schneider, T., G. Grosbois, W. F. Vincent, and M. Rautio. Saving for the future: Fall uptake of algal lipids supports copepod egg production in spring. Submitted manuscript.

**III.** Schneider, T. and M. Rautio. Copepods reduce carotenoid pigmentation at warm ambient temperature independently of UVR exposure. Manuscript.

In Chapter I we assessed the seasonal pattern of copepod carotenoid content and evaluated several candidate driving factors to explain the seasonal changes. The copepods contained mostly astaxanthin (>99% of total carotenoids). Due to the pronounced pigmentation maximum in winter, photoprotection was ruled out as a primary function of carotenoids in this copepod population. We found that the esterified fractions of copepod astaxanthin were strongly correlated with the animals' fatty acid reserves. We have developed a measure of the rate of change of copepod carotenoid content that has allowed us to relate these changes to dietary constraints and reproductive effort.

In Chapter II we combined data on the seasonal changes in copepod carotenoids and fatty acids with measures of primary productivity and copepod reproduction investment to obtain a quantitative estimate of the trophic transfer of both carotenoids and selected fatty acids from phytoplankton to copepods and the subsequent transfer

to copepod eggs. The idea of this chapter was to follow the pathway of these compounds within the lake ecosystem and to evaluate whether potential dietary constraints imposed on copepods would result in similar seasonal patterns of reserve accumulation and spending of both carotenoids and fatty acids. We found that phytoplankton productivity during winter is too low to sustain copepod reproduction. Copepods are able to reproduce using their carotenoid and fatty acid reserves, a significant part of which is invested into eggs. Furthermore we show that in contrast to the open-water period, during winter copepods contain more astaxanthin and  $\omega$ 3 fatty acids than the lake seston per unit volume.

In Chapter III, I present the results of two laboratory experiments conducted in order to elucidate the potential roles of UVR and temperature for the accumulation of carotenoids by copepods. Copepods exposed to natural levels of UVR during 18 to 21 days did not change their content of carotenoids as compared to the control groups (no light, visible light only). Neither did mortality differ according to the light or UVR treatment. However, water temperature had a strong effect on both survival and carotenoid content, with less surviving individuals at 15°C that also had lower final carotenoid content than the ones kept at 5°C.

# I. SEASONAL DRIVERS OF COPEPOD CAROTENOID ACCUMULATION

PUBLISHED RESEARCH ARTICLE

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

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Running head: Copepod carotenoids and lipids

Keywords: zooplankton, astaxanthin, astaxanthin esters, fatty acids, oxidative stress, antioxidant, ultraviolet radiation, visual predation.

## 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 *Leptodiaptomus minutus* along with UVR exposure, water temperature, phytoplankton pigments, and fish predation in a boreal lake during eighteen 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 ( $-0.018$  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.

## Introduction

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 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 2014), 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  $\beta,\beta$ -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 the present 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 and 8.3 mg C L<sup>-1</sup> and the photic zone reaches the bottom. The crustacean zooplankton community of the lake principally consists of six copepod species (*Leptodiaptomus minutus*, *Epischura lacustris*, *Aglaodiaptomus spatulocrenatus*, *Cyclops scutifer*, *Mesocyclops edax*, *Tropocyclops prasinus*) and

five cladocerans (*Bosmina longirostris*., *Daphnia* spp., *Diaphanosoma 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.

#### *Leptodiptomus* 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 4 December 2011 to 7 May 2013. When the lake was ice-covered, sampling was conducted through a hole (diameter *ca.* 40 cm). Zooplankton was sampled by vertical net tows (diameter: 24 cm; mesh size: 50  $\mu$ m) 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  $\mu$ m sieve and kept overnight at either 5°C (in winter) or 15°C (in summer) for gut evacuation. On the following day, adult *Leptodiptomus minutus* were individually picked from CO<sub>2</sub>-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 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  $-80^{\circ}\text{C}$  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, Wiarnton, Ontario) on ice and then sonicated for three times 20 s on ice at 10 W using a rod sonicator (Microson XL2000, Misonix, Farmingdale, NY) . This protocol enabled optimal extraction of carotenoids from zooplankton samples (Rautio et al. 2009). The extracts were incubated overnight at  $-20^{\circ}\text{C}$  under argon atmosphere, then centrifuged and filtrated through 0.2  $\mu\text{m}$  polytetrafluorethylene membrane filters (VWR international, Mississauga, Ontario, Canada) and stored at  $4^{\circ}\text{C}$  in the dark under argon gas until HPLC analysis within 48 h. 50  $\mu\text{L}$  were injected into an Accela 600 HPLC system (Thermo Scientific, Waltham, MA, U.S.A.) equipped with a Hypersil Gold C8 column (150 mm  $\times$  4.6 mm, 3  $\mu\text{m}$  particle size, Thermo Scientific) protected by a Hypersil Gold C8 guard column (10 mm  $\times$  4 mm, 3  $\mu\text{m}$  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 2014) by separating the first and second clusters of peaks.

#### *UVR exposure and temperature*

High-resolution vertical profiles of ultraviolet radiation (UVR) and temperature were obtained with a PUV-2500 profiler radiometer (Biospherical Instruments, San Diego, CA, 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  $0.1 \text{ m s}^{-1}$ , 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 ( $E_d$ ) versus 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 2 April 2013, where an aluminum arm was used to position the radiometer *c.* 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:

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

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

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

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://zardozi.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:

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

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

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

where  $\Delta Z = Z - Z_{ice^-}$ , *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 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  $\mu\text{m}$  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  $-80^{\circ}\text{C}$  until freeze-drying, after which they were stored at  $-50^{\circ}\text{C}$ . 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  $\text{H}_2\text{SO}_4$ -methanol (1% v/v) was added to promote transesterification at  $50^{\circ}\text{C}$ ; the resulting fatty acid methyl esters (FAMES) were separated from non-FAME components by addition of  $\text{KHCO}_3$ -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, CA) 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  $\mu\text{m}$  film thickness). Helium was used as the carrier gas (flow rate 1 mL  $\text{min}^{-1}$  with electronic pressure control) and the temperature ramp was as follows: 70°C for 1.5 min followed by an increase of 20°C  $\text{min}^{-1}$  until 110°C, an increase of 12.5°C  $\text{min}^{-1}$  until 160°C, and an increase of 2.5°C  $\text{min}^{-1}$  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) versus 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 (50x 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*, *S. margarita*, *Notropis atherinoides* and *N. 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 2013 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 brook trout is limited by water temperature (highest between  $15\text{ }^{\circ}\text{C}$  and  $22\text{ }^{\circ}\text{C}$ ) 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 versus 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.

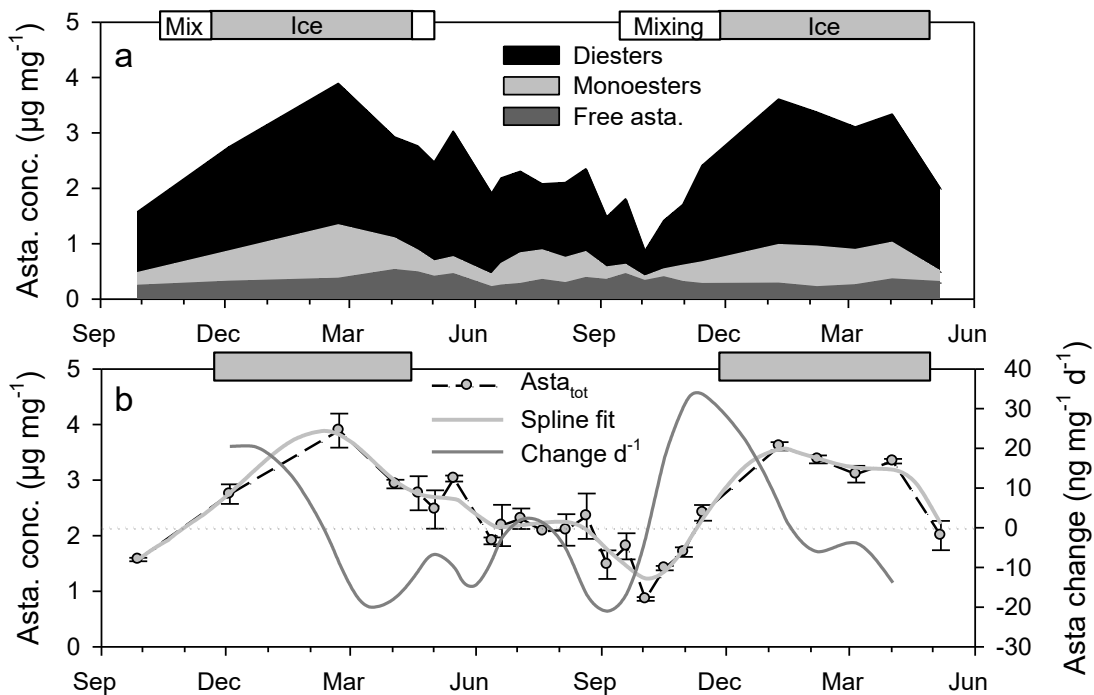
Pairwise correlations were calculated between *Leptodiaptomus* astaxanthin (mono- and diesters, free astaxanthin, total content and rate of change) and environmental variables. We applied multiple linear regression analysis to identify the best explanatory variables (temperature, diet, fatty acids, reproduction 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 ( $\beta,\beta$ -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  $\Delta AIC_c$ , 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; 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, NC, 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 3 October 2012; Fig. I.1a). During the following three months, copepod astaxanthin content increased four-fold to reach a winter maximum of  $3.61 \pm 0.08 \mu\text{g mg}^{-1}$  on 9 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 ( $\text{ng ind}^{-1}$ ) ( $t$ -test,  $p < 0.001$ ) and both measures of carotenoids were strongly correlated ( $p = 0.93$ ,  $p < 0.001$ ). The rate of change in  $Asta_{tot}$  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 ( $-17.6$  and  $-21.0 \text{ ng mg}^{-1} \text{ d}^{-1}$ , respectively; Fig. I.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.



**Figure I.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.

The carotenoids in copepods were comprised mainly ( $> 99\%$ ) of astaxanthin in three forms: free astaxanthin, monoesters and diesters, complemented by traces of alloxanthin and  $\beta,\beta$ -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 ( $\text{Asta}_{\text{tot}}$ ; hereafter used synonymously for total carotenoids per

dry mass) in *L. minutus* (Fig. I.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. I.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 3 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 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. I.2a). Likewise, water temperature and seston pigment concentration were low during winter but increased rapidly after ice breakup (Fig. I.2a). Phytoplankton pigments were dominated by chlorophyll *a*, followed by fucoxanthin, zeaxanthin, alloxanthin, chlorophyll *b*, violaxanthin,  $\beta,\beta$ -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,  $\beta,\beta$ -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 chlorophyll *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. I.2b).

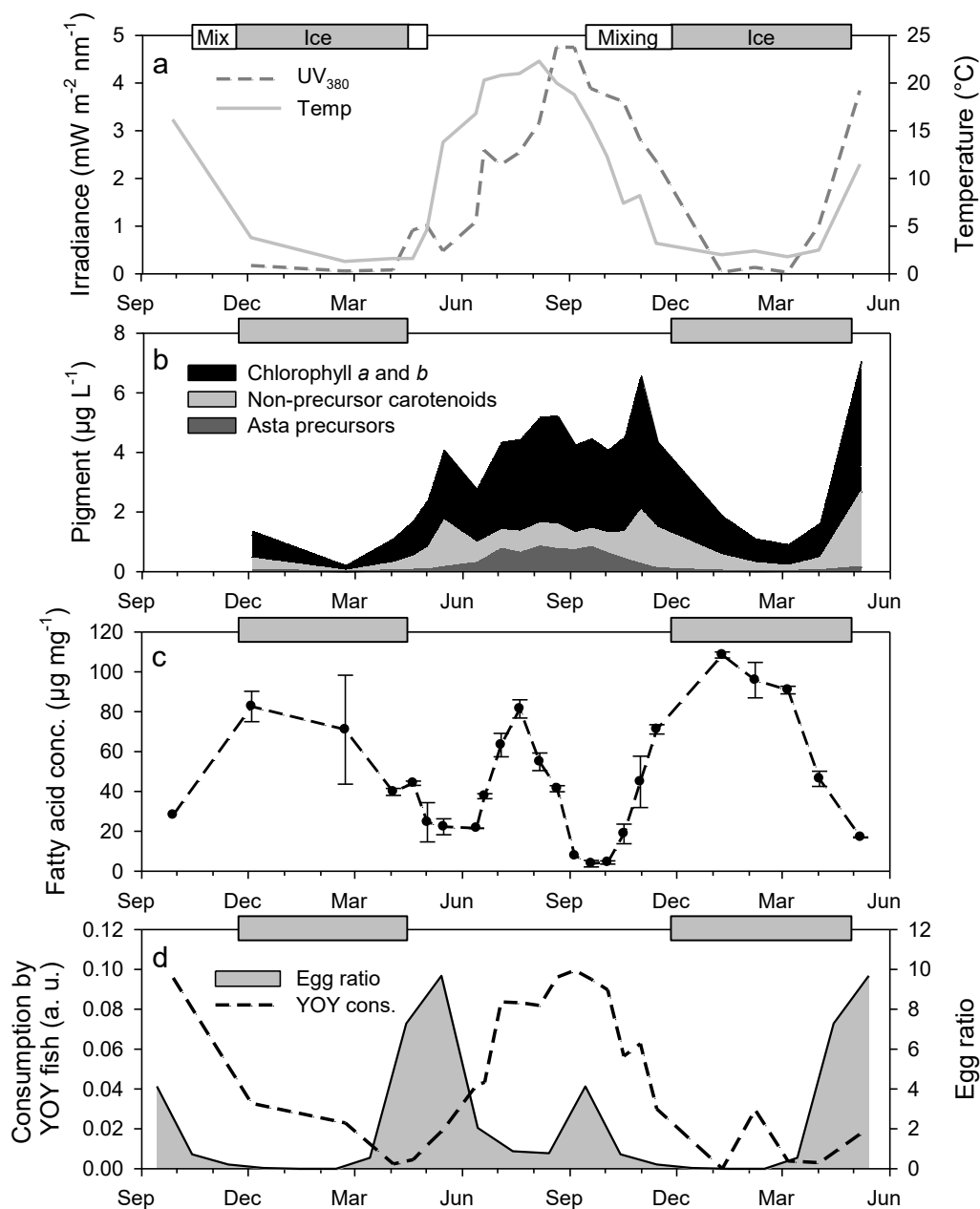


Figure I.2 (caption on following page)

**Figure I.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.

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. I.2c). This peak partly coincided with the presence of juvenile stages in the samples from 13 June 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) 47% 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. I.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. I.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 I.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 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 *Leptodiatomus* egg ratio ( $r = -0.50$ ,  $p = 0.017$ ) but was unrelated to other environmental variables (Table I.1).

Multiple linear regression (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 I.2; Fig. I.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 I.2).

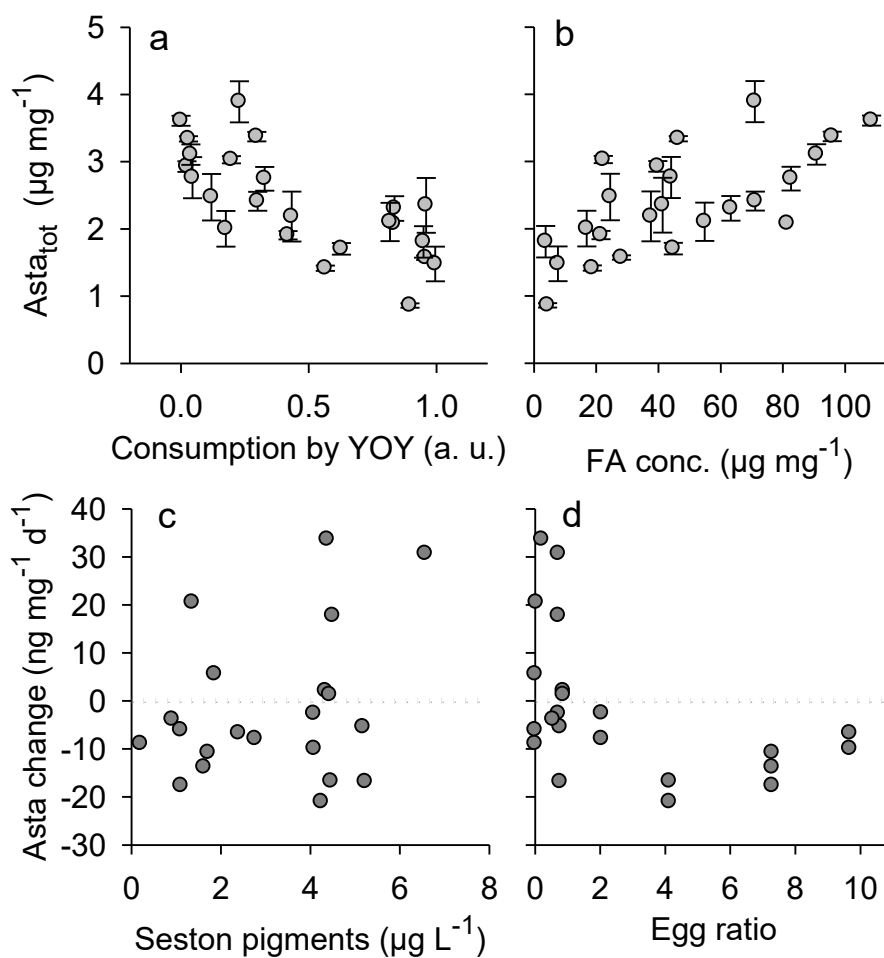


**Table I.1** Correlation with potential explanatory variables of total astaxanthin and rate of change of this pigment in the copepod *Leptodiaptomus minutus* sampled in Lake Simoncouche. UV<sub>380</sub>, irradiance of UVR at 380 nm at 1 m depth; Temperature, water temperature at 1 m depth; Precursors, sum of potential astaxanthin precursors ( $\beta,\beta$ -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+1)-transformed egg ratio; FA<sub>tot</sub>, 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 <sub>380</sub>	-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 <sub>tot</sub>	0.69	24	<0.001	0.34	22	0.124

For Asta change, MLR revealed four models with  $\Delta AIC_c < 2$  (Table I.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. I.3c, d), while copepod fatty acid concentration was of low importance for variation in Asta change.



**Figure I.3** 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.

**Table I.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 I.1. Relative variable importance (RVI) below each predictor variable,  $P$ -value and semipartial  $R^2$  ( $SR^2$ ) below the standardized regression coefficient ( $Std\ \beta$ ), total  $R^2$  and adjusted  $R^2$ , mean square error (MSE),  $\Delta AIC_c$  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	$\Delta AIC_c$	$w_i$
$Asta_{tot}$	RVI	0.24	0.66	0.84	0.28	0.95				
A1	$Std\ \beta$		-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	$Std\ \beta$			-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	$Std\ \beta$	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	$Std\ \beta$		-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	$Std\ \beta$		-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				

Table I.2 (continued)

Model		Temp	Food	YOY cons.	Eggs	FA <sub>tot</sub>	R <sup>2</sup> (adj. R <sup>2</sup> )	MSE	ΔAIC <sub>c</sub>	w <sub>i</sub>
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 <sup>2</sup>		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 <sup>2</sup>	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	<.0001	0.1024	0.0017		(0.67)			
	SR <sup>2</sup>	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	<.0001			0.0098	(0.60)			
	SR <sup>3</sup>	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 <sup>2</sup>	0.43	0.27		0.24	0.01				

## 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 differed 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. For the duration of winter, the water column was characterized by highly pigmented copepods whose elevated astaxanthin concentration 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 photoprotective 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 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 2014). It has also been speculated that astaxanthin may serve as a physiological replacement for molecular oxygen, allowing for rapid utilization of energy reserves (Łotocka et al. 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 the free form rather than as esters (Łotocka et al. 2004). These observations further underline the dynamic use and re-allocation 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 et al. 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 et al. 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,  $\beta,\beta$ -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 pre-winter 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 non-enzymatic 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  $3^{\circ}\text{C}$  and  $10^{\circ}\text{C}$ . 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 multiple linear regression 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.

## **References**

The references of each individual chapter have been included in the reference section at the end of the thesis.

## *Acknowledgments*

We thank Lars-Anders Hansson for valuable discussion, comments and suggestions on several versions of the manuscript, Takehito Yoshida and three anonymous reviewers for constructive and insightful comments, Sonya Levesque and Pierre Magnan for their advice with fish calculations, Mathieu Cusson for support with statistics, and David-Alexandre Gauthier, Marie-Josée Martineau and Denis Walsh for field and laboratory assistance. This research was financed by the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, and the Fonds québécois de la recherche sur la nature et les technologies.

## II. TRANSFER OF CAROTENOIDS AND FATTY ACIDS FROM PHYTOPLANKTON TO COPEPOD EGGS

MANUSCRIPT IN REVIEW

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

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Running head: Trophic transfer of carotenoids and fatty acids

Keywords: zooplankton, carotenoids, astaxanthin, omega-3, n-3, fatty acids, reproduction, winter

## Summary

1. The freshwater copepod *Leptodiaptomus minutus* has its main annual reproductive period at the end of winter following months of ice-cover and limited primary production. Yet the copepods transfer large quantities of previously accumulated astaxanthin carotenoids and fatty acids to their eggs, presumably to provide the offspring with antioxidant protection and energy reserves.
2. We hypothesized that the seasonal dynamics of copepod pigmentation and lipid content result from a capital breeding strategy allowing the animals to produce offspring in time for the nauplii to feed on the spring phytoplankton bloom, thus gaining a competitive advantage. We quantified the production of astaxanthin precursor carotenoids and  $\omega$ 3 fatty acids by the phytoplankton as well as the amount required for egg production and estimated the transfer rates from the phytoplankton to copepod eggs.
3. 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. High rates of lipid reserve accumulation in *L. minutus* in late autumn and early winter accounted for an appreciable portion of the daily production by the phytoplankton underlining the importance of pre-winter primary production for reserve-building in this copepod.
4. During winter, the sum of astaxanthin and its precursors as well as the sum of  $\omega$ 3 fatty acids stocked in copepods exceeded the respective concentrations in the lake seston, underlining the importance of reserve accumulation at the ecosystem level. Consequently, adult copepods act as a lipid storage pool linking the biosynthesis of carotenoids and fatty acids

by primary producers in autumn to the production of copepod eggs at the end of winter.

## **Introduction**

One of the key life-history traits that contributes 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 accumulated ahead of time (Varpe 2012; Maps et al. 2013). In addition to storage lipids, copepods often have higher concentrations of carotenoids during winter than in summer (Hairston 1979a; Hansson 2004; García et al. 2008), and both classes of compounds may be subsequently transferred to eggs (Schneider et al. 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 et al. 2009; Varpe et al. 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 that zooplankton cannot synthesize *de novo* and thus need to acquire from their diet. For example, copepods are able to convert their major carotenoid, astaxanthin, from phytoplanktic precursors such as zeaxanthin,  $\beta,\beta$ -carotene and potentially lutein (Rhodes 2006; Caramujo et al. 2012). Carotenoids in copepod eggs may provide photoprotection to nauplii (Hairston 1979b) but have also

been suggested to stimulate naupliar metabolism via physiological replacement of oxygen allowing for rapid combustion of energy reserves (Łotocka et al. 2004).

In the case of fatty acids (FAs), essentiality is a more complex subject. All animals including crustaceans require specific combinations of polyunsaturated FAs (PUFAs) to grow, develop and reproduce. For example, feeding experiments have shown that cladocerans need algal derived PUFAs for survival, growth and reproduction (Wenzel et al. 2012). More precisely, *Daphnia* specifically retain  $\omega$ 3 FAs, and a high ratio of  $\omega$ 3:  $\omega$ 6 FAs reflects good nutritional conditions (Taipale et al. 2015). Additionally, high dietary concentrations of docosahexaenoic acid (DHA; 22:6 $\omega$ 3) may promote egg production in copepods (Evjemo et al. 2008), while eicosapentaenoic acid (EPA; 20:5 $\omega$ 3) and arachidonic acid (ARA; 20:4 $\omega$ 6) are important precursors for eicosanoids, a group of signal molecules (Brett and Müller-Navarra 1997), and stearidonic acid (SDA; 18:4n-3) is associated with increased winter survival (Mariash et al. 2016). Many animals can convert some C<sub>18</sub> PUFAs such as  $\alpha$ -linolenic acid (ALA; 18:3 $\omega$ 3) or linoleic acid (LIN; 18:2 $\omega$ 6) via elongation and desaturation to obtain long-chain PUFAs such as DHA, EPA or ARA, albeit with limited efficiency. Although this ability varies among copepods and is not known for each species, crustaceans generally cannot synthesise  $\omega$ 3 or  $\omega$ 6 FAs *de novo*. Therefore, the total pool of  $\omega$ 3 and  $\omega$ 6 PUFAs, respectively, is 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 investigate 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 smaller second peak at the end of summer. We first estimated the production of astaxanthin precursor carotenoids and  $\omega$ 3 FAs in the seston for one complete year. These groups of compounds were chosen due to their essential role in zooplankton nutrition; i.e., 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  $\omega$ 3 precursors, we looked at the sum of  $\omega$ 3 PUFAs as a whole. 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 which extent the above mentioned sources contribute to copepod reproduction. Additionally, we determined the standing stocks of astaxanthin and  $\omega$ 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 secondary production. We hypothesized that accumulation of astaxanthin and  $\omega$ 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 et al. 2011) that precedes their annual main reproductive period. Instead, we expected to find that these animals live on their reserves throughout the winter and produce eggs predominantly from stored reserves, including storage lipids and carotenoids.

## Methods

### *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 ( $Z_{\text{mean}} = 2.2$  m,  $Z_{\text{max}} = 8$  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. 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 and 8.3 mg C L<sup>-1</sup> 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 consists of six copepod species (*Leptodiaptomus minutus*, *Epischura lacustris*, *Aglaodiaptomus spatulocrenatus*, *Cyclops scutifer*, *Mesocyclops edax*, *Tropocyclops prasinus*) and five cladocerans (*Bosmina* spp., *Daphnia* spp., *Diaphanosoma* spp., *Holopedium glacialis*, *Leptodora kindtii*). *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 unpubl. 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.

### *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 m or 1.5 m) from the whole water column and 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, 40x magnification). Females were identified according to the morphology of their P5 legs (Stratton Wilson and 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 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 fatty acids 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. When the lake was ice-covered, sampling was conducted through a hole. Zooplankton was collected by vertical net tows (diameter: 24 cm; mesh size: 50  $\mu$ m) 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  $\mu$ m 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 *Leptodiaptomus minutus* were individually picked from CO<sub>2</sub>-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 2700 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 analysis (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 to 200 individuals, with three sub-samples per analysis. The animals were transferred into 1.5 mL-plastic tubes and stored at  $-80^{\circ}\text{C}$  until they could be freeze-dried. Dry mass of adult copepods was determined on a Mettler Toledo XP26 microbalance ( $\pm 2 \mu\text{g}$ ; Mettler Toledo, Columbus, Ohio, United States) from three freeze-dried sub-samples per sampling date, each containing 100 to 200 individuals. Egg dry mass was determined as the weight (same microbalance) of each freeze-dried egg sample, divided by the number of eggs, and averaged over all samples to obtain a reliable estimate. This value ( $0.13 \pm 0.05 \mu\text{g egg}^{-1}$ ) was close to the one based on a volumetric estimate ( $0.15 \mu\text{g egg}^{-1}$ ), which was derived from carbon content (Huntley and Lopez 1992) assuming  $0.4 \mu\text{g C per } \mu\text{g}$  of dry mass.

Water destined for the assessment of seston pigments and fatty acids was collected on the same dates as copepods at regular depth intervals (either 1 m or 1.5 m) including a sub-surface sample using the Limnos water bottle; water samples were integrated over each layer (epilimnion and, when the lake was stratified, metalimnion). The

water was prefiltered through a 50  $\mu\text{m}$  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  $-80^{\circ}\text{C}$  until freeze-drying, after which they were stored at  $-50^{\circ}\text{C}$ .

#### *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, homogenized for 2 min (Caframo R2R1 tissue grinder, Wiarion, Ontario, Canada) on ice and then sonicated as described above. This protocol enabled optimal extraction of carotenoids from zooplankton samples (Rautio et al. 2009). The extracts were incubated for 30 min on ice (seston) or overnight at  $-20^{\circ}\text{C}$  (zooplankton) under argon atmosphere, then centrifuged and filtered through 0.2  $\mu\text{m}$  polytetrafluorethylene membrane filters (VWR international, Mississauga, Ontario, Canada) and stored at  $4^{\circ}\text{C}$  in the dark under argon gas until HPLC analysis within 48 h. 50  $\mu\text{L}$  were injected into an Accela 600 HPLC system (Thermo Scientific, Waltham, MA, U.S.A.) equipped with a Hypersil Gold C8 column (150 mm  $\times$  4.6 mm, 3  $\mu\text{m}$  particle size, Thermo Scientific) protected by a Hypersil Gold C8 guard column (10 mm  $\times$  4 mm, 3  $\mu\text{m}$  particle size, Thermo Scientific) using the HPLC protocol of Zapata et al. (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 et al. 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.

#### *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 (4000 : 1000 : 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 and 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  $\mu\text{m}$  film thickness). Helium was used as the carrier gas (flow rate 1  $\text{mL min}^{-1}$  with electronic pressure control) and the temperature ramp was as follows: 70°C for 1.5 min followed by an increase of 20°C  $\text{min}^{-1}$  until 110°C, an increase of 12.5°C  $\text{min}^{-1}$  until 160°C, and an increase of 2.5°C  $\text{min}^{-1}$  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) versus an internal standard (nonadecanoic acid) using calibration curves based on

known standard concentrations (see Appendix S2 for detailed information on the standards used).

### *Primary productivity*

The productivity of pelagic algal assemblages was quantified from  $^{14}\text{C}$  uptake in multiple incubations under a gradient of irradiance conditions on five dates in 2012 and on eight occasions in 2014/15. Integrated water samples containing natural densities of phytoplankton were taken from epilimnion and metalimnion (when present), sieved through a 50  $\mu\text{m}$  net to remove grazers and filled into 20-mL glass scintillation vials. A sample of raw water was kept in an air-tight 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  $\mu\text{L}$  of  $^{14}\text{C}$  solution (80  $\mu\text{Ci ml}^{-1}$ ) were added, and after thorough mixing, subsamples of 200  $\mu\text{L}$  were taken for total activity assessment and placed in plastic scintillation vials already containing 200  $\mu\text{L}$  of ethanolamine. The glass vials were incubated for 1–2 h 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 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 h after incubation, and 0.25 mL HCl (0.5 N) was added to remove excess  $^{14}\text{C}$ .

5 mL 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  $\text{mg C m}^{-3} \text{ h}$ ) was then calculated as

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

where  $dpm$  is counts per minute of either sample or total activity, DIC is the dissolved inorganic carbon concentration ( $\text{mgC m}^{-3}$ ) 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 ( $0.2 \times 10^{-3}$  L), respectively, 1.05 the isotopic discrimination factor correcting for slower uptake of the heavier isotope, and  $time$  is the incubation time in h.

Primary productivity ( $PP$ ; average values of two replicate incubations) were fitted by a regression following Platt et al. (1980) to solve the photosynthetic parameters  $p$ ,  $\alpha$ , and  $\beta$ :

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

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

#### *Calculations and data analysis*

Several sets of data obtained in 2011–2015 were combined to evaluate the annual pattern of copepod abundance and life stages, primary production of pigments and fatty acids, 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 fatty acid production in the lake seston were obtained as a function of primary productivity (i.e., production per day) and pigment and fatty acid concentration. In a first step, water-column PAR irradiance was calculated on each sampling date for each hour of the day in each meter 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). Primary productivity ( $PP$ , eq. 2) was then modelled as a function of PAR using the photosynthetic parameters  $p$ ,  $\alpha$ , and  $\beta$  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 primary productivity in each meter of depth was multiplied by a depth weighing factor accounting for the bathymetry of the lake; i.e., 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 epi- 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  $\beta,\beta$ -carotene) and the sum of  $\omega 3$  FAs on that date to obtain a production estimate for precursor pigments and  $\omega 3$  FAs in the lake seston. The production rates and standing stocks in the epi- 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  $\omega 3$  FAs ( $\text{ng ind}^{-1} \text{d}^{-1}$ ) was estimated from seasonal changes in copepod content of the respective compounds as in Schneider et al. (2016). In brief, a penalized cubic regression spline was fitted on copepod astaxanthin and  $\omega 3$  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 normalized to the unit volume by multiplying them with copepod abundance.

The transfer rates of astaxanthin and fatty acids from adult copepods to eggs were estimated based on egg production rate on a given date combined with egg-specific contents of astaxanthin and  $\omega$ 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  $a = 349637$ ,  $\alpha = -9.61$  and  $b = -2.51$ . Daily egg production was then calculated as  $P_{\text{egg}} = 24 \cdot \text{egg abundance} / D$ . This value was multiplied by the egg-specific carotenoid and fatty acid contents to obtain the transfer rates of these compounds from copepods to eggs.

## Results

### *Seasonal cycle of Leptodiaptomus minutus*

The high sampling frequency of copepod seasonal abundance allowed for precise temporal allocation of each life stage (Fig. II.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 (Fig. II.1 a–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

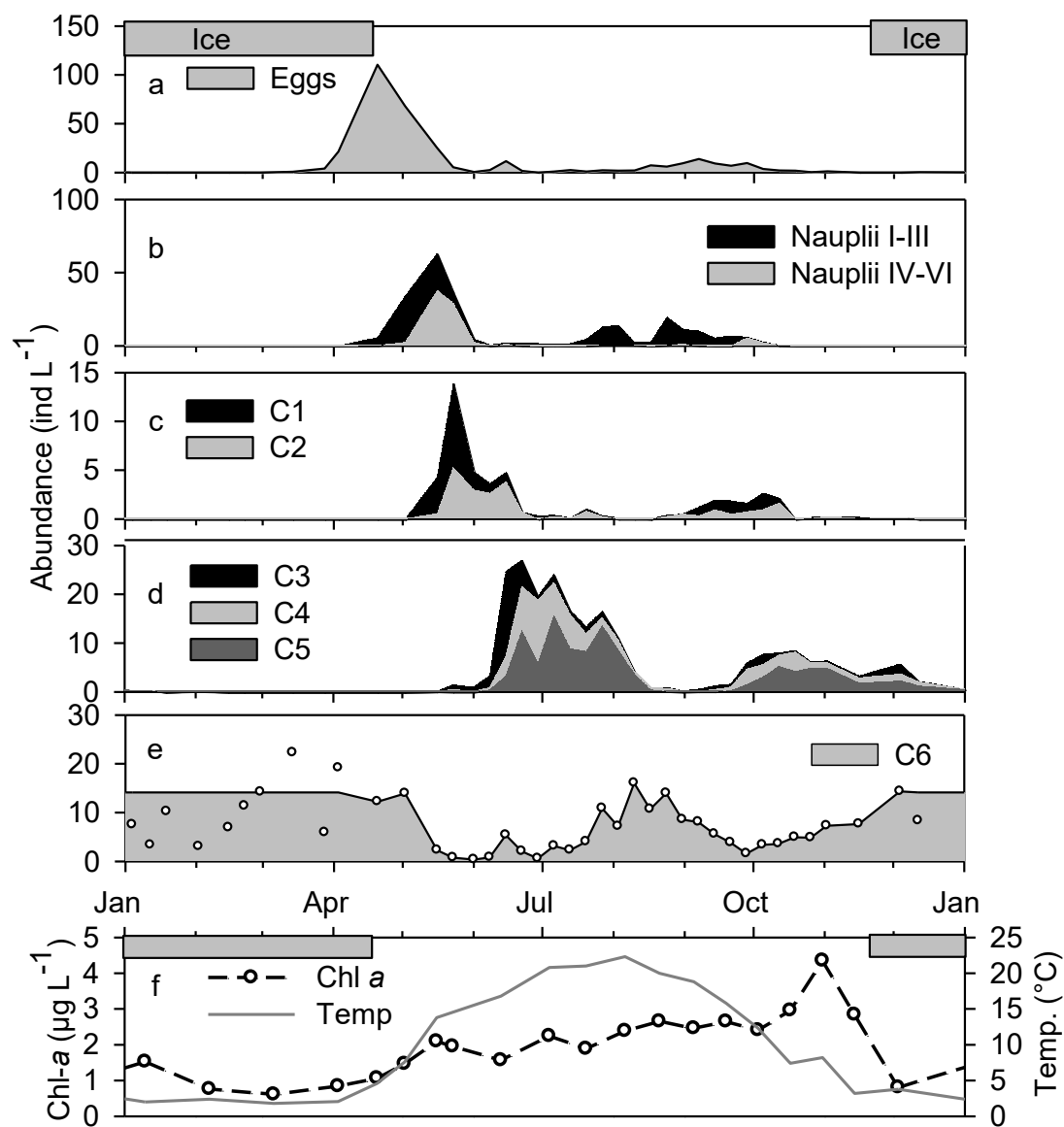


found in the water column as water temperature and chlorophyll concentration reached their annual minima (Fig. II.1 e–f).

**Table II.1** Sampling periods of different sets of 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

*L. minutus* eggs were most abundant in April (110 eggs L<sup>-1</sup>) while the second peak in September was much lower (14 eggs L<sup>-1</sup>; Fig. II.1a). However, egg production rates, as estimated based on temperature-dependent development time, had more similar maximum values in spring (6.3 eggs L<sup>-1</sup> day<sup>-1</sup>) and in summer (3.6 eggs L<sup>-1</sup> day<sup>-1</sup>). Due to the longer reproduction period in summer (119 d compared to 100 d in winter-spring), the estimated numbers of total eggs produced by each cohort were 242 eggs L<sup>-1</sup> from March to June, and 146 eggs L<sup>-1</sup> from July to October.



**Figure II.1** Annual population dynamics of *L. minutus* (a–e) and seasonal changes in Chl *a* and temperature (f) in Lake Simoncouche. Two generations can be distinguished according to the progression of developmental stages (a–e). During the winter period, adult abundance has been smoothed using an average winter abundance value (e).

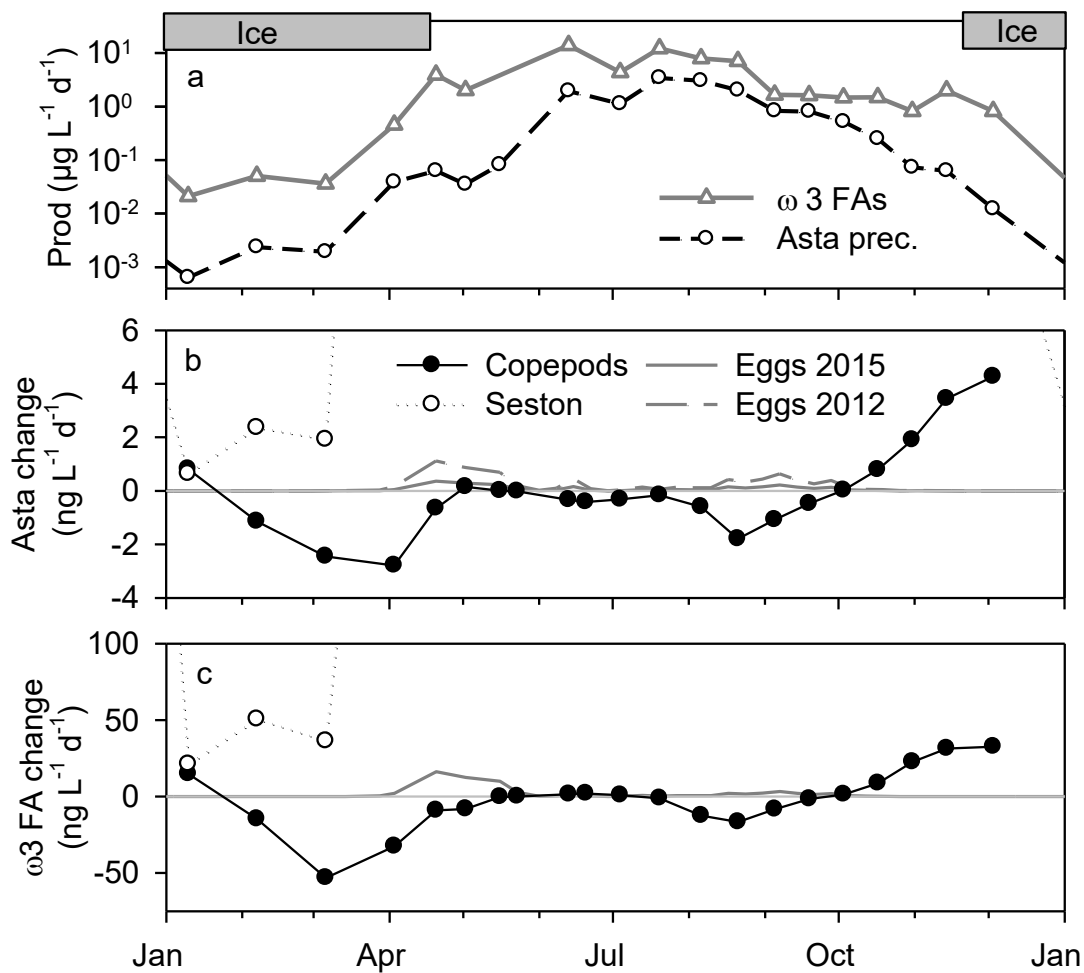
*Production of astaxanthin precursors and fatty acids in phytoplankton*

The concentrations of the three astaxanthin precursor carotenoids present in the seston (lutein, zeaxanthin and  $\beta,\beta$ -carotene) were strongly correlated to each other ( $r > 0.9$ ) and their estimated production did thus follow the same seasonal pattern (Fig. II.2a). In general, zeaxanthin was most abundant, followed by  $\beta,\beta$ -carotene and lutein. Astaxanthin precursor productivity (all three pigments summed together) was low when the lake was ice-covered ranging from  $0.0006 \mu\text{g L}^{-1} \text{d}^{-1}$  in January to  $0.04 \mu\text{g L}^{-1} \text{d}^{-1}$  in April (Fig. II.2a). Precursor productivity then increased to its maximum values of  $3.4 \mu\text{g L}^{-1} \text{d}^{-1}$  in July-August. During the autumnal mixing period the productivity of astaxanthin precursors decreased from  $0.8 \mu\text{g L}^{-1} \text{d}^{-1}$  in September to  $0.06 \mu\text{g L}^{-1} \text{d}^{-1}$  in November. Similar to the astaxanthin precursor carotenoids, seston productivity of the sum of  $\omega 3$  FAs was low during most of the winter ( $< 0.1 \mu\text{g L}^{-1} \text{d}^{-1}$  from January to March) but was characterized by distinct productivity peaks in early spring and in late autumn ( $3.9 \mu\text{g L}^{-1} \text{d}^{-1}$  in April and  $2 \mu\text{g L}^{-1} \text{d}^{-1}$  in November) and by a comparably less pronounced mid-summer maximum of  $14 \mu\text{g L}^{-1} \text{d}^{-1}$  in June (Fig. II.2a).

*Carotenoid and fatty acid content in copepod adults and eggs*

The rate of change in copepod carotenoid content showed a three-month period of net accumulation from October to January (positive rate of change, Fig. II.2b–c) and two periods of net loss (negative rate of change), one from February to April and another one in August-September (Fig. II.2b). The rate of change in the sum of  $\omega 3$  FAs showed the same general pattern (Fig. II.2c). The periods of loss shortly preceded the timing of egg production suggesting that astaxanthin and fatty acids were transferred to the eggs in the course of reproduction (Schneider et al. 2016). The net uptake rates of both astaxanthin and  $\omega 3$  FAs reached their maximum in early December:  $0.45 \text{ ng ind}^{-1} \text{ d}^{-1}$  of astaxanthin and  $4.11 \text{ ng ind}^{-1} \text{ d}^{-1}$  of  $\omega 3$  FAs. Normalized to the water

volume, the maximum uptake rates per individual copepod were  $4.3 \text{ ng L}^{-1} \text{ d}^{-1}$  for astaxanthin, and  $32.6 \text{ ng L}^{-1} \text{ d}^{-1}$  for  $\omega 3$  FAs (Fig. II.2b, c).



**Figure II.2** Production of astaxanthin precursors and  $\omega 3$  fatty acids in the lake seston (a), change in copepods and transfer to eggs of astaxanthin (b) and  $\omega 3$  FAs (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).

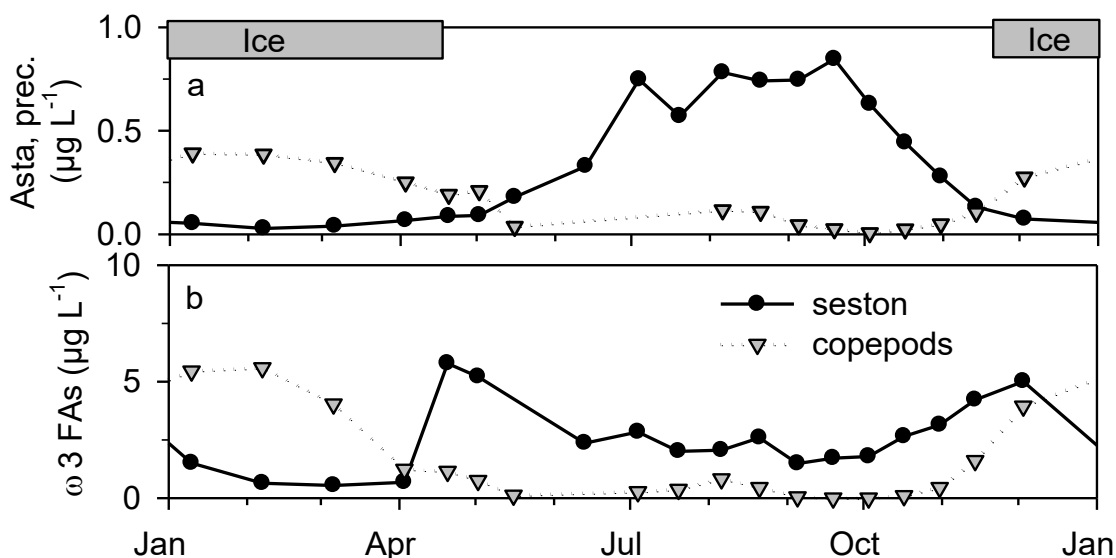
Phytoplankton productivity of astaxanthin precursors and fatty acids largely exceeded the demand by adult copepods as determined from net accumulation rates during the open-water period. However, copepod net uptake rates of astaxanthin precursors and of  $\omega$ 3 FAs in January ( $0.81$  and  $14.7 \text{ ng L}^{-1} \text{ d}^{-1}$ ) were in the same range than the respective phytoplankton productivity of these compounds ( $0.63$  and  $21.1 \text{ ng L}^{-1} \text{ d}^{-1}$ ), which remained relatively low until March (Fig. II.2b-c).

There were large variations in the carotenoid content of the copepod eggs. The two samples in May 2012 contained  $0.51$  and  $0.27 \text{ ng egg}^{-1}$  of astaxanthin, while in 2015 egg astaxanthin content was only  $0.03$  (May) and  $0.05$  (September)  $\text{ng egg}^{-1}$ . This difference was largely due to a much lower content of esterified astaxanthin in eggs, which accounted (mono- and diesters combined) for 29-32% in 2015 as compared to 76-77% in 2012. Egg total FA content was  $3.2$  and  $9.6 \text{ ng egg}^{-1}$  in May and  $7.5 \text{ ng egg}^{-1}$  in September. The percentage of  $\omega$ 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 fatty acids from the copepods to their eggs. These transfer rates were highest during ice-off at the peak of egg production in April (Fig. II.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 (Fig. II.2b). Egg production during the same period amounted to  $38 \text{ ng astaxanthin L}^{-1}$ , which was 20% of the loss in copepods. In the summer cohort, astaxanthin was reduced by  $64 \text{ ng L}^{-1}$  from July to September corresponding to  $26 \text{ ng L}^{-1}$ , 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.  $\omega$ 3 FA reduction in copepods was  $2.02 \mu\text{g L}^{-1}$  in winter and  $0.57 \mu\text{g L}^{-1}$  in summer, corresponding to  $\omega$ 3 FA investment into egg production of  $0.56 \mu\text{g L}^{-1}$  (28%) in winter and  $0.13 \mu\text{g L}^{-1}$  (23%) in summer, respectively. However, since our samples included females and males, the role of egg

investment may have been underestimated (female:male ratio was *c.* 40:60 on average).

*Distribution of astaxanthin and fatty acid stocks in plankton*

Copepods served as an important storage of carotenoids and fatty acids 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  $0.4 \mu\text{g L}^{-1}$  vs.  $0.04 \mu\text{g L}^{-1}$  in March; Fig. II.3a). Similarly, from December to April,  $\omega 3$  FAs were more abundant in copepods than in the seston (e.g.,  $\omega 3$  FAs in March:  $5.6 \mu\text{g L}^{-1}$  in copepods vs.  $0.54 \mu\text{g L}^{-1}$  in the seston; Fig. II.3b).



**Figure II.3** Allocation of astaxanthin and its precursors as well as  $\omega 3$  FAs in lake seston and copepods.

## Discussion

Our results provide evidence of capital breeding in the boreal copepod *Leptodiaptomus minutus* reproducing under ice with a strong link between investment of astaxanthin and fatty acids in eggs and the concurrent decline of these lipid reserves in adult copepods. The astaxanthin pigmentation and fatty acid content of copepods in this lake changed markedly with season and did not track the respective concentrations in the seston diet, where these compounds for most part of the year were in excess. Instead, the period of most pronounced astaxanthin and fatty acid accumulation 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 and  $\omega$ 3 FAs in the lake seston, changes in astaxanthin and  $\omega$ 3 FA content in copepods were correlated. Their concurrence 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 and Schnack-Schiel 1996). Some polar marine copepods may graze on sedimenting sea-ice algae to support reproduction (Runge et al. 1991), but this option appears to be limited to certain marine systems providing appropriate physical conditions (Hirche and Kosobokova 2003). Lake ice lacks the brine channels and their associated high concentrations of diatoms that characterize sea-ice, and we are not aware of any evidence of ice algae contribution to secondary production in freshwaters. 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 fatty acids 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 et al. 1980; Wærvågen and Nilssen 2010; Rautio et al. 2011). The small egg peak in June (Fig. II.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 four months in spring-summer and two to three months in autumn (Fig. II.1a–e). The comparison with water temperature and chlorophyll *a* suggests that the winter-spring egg peak is timed so that the new cohort of nauplii will reach their feeding stage in time to utilize the increasing productivity of the lake as temperature rises (Fig. II.1f).

Phytoplankton production rates of astaxanthin precursors and  $\omega$ 3 FAs differed by two to three orders of magnitude in summer and winter, respectively. From January to March, primary productivity was relatively low. Seston productivity, however, started to increase already before ice-off. Thus, at egg spawning time (April and May) there was no apparent limitation of resources (FAs or carotenoids) that were required for egg production. However, it must be taken into account that the eggs need to develop in the female copepods' ovaries before being spawned. Oocyte maturation strongly depends on ambient temperature and generally takes an amount of time comparable to the embryonic development (Caramujo and Boavida 1999). There is little information about *L. minutus* in this regard, however data are available for other diaptomid copepod species. Two alpine diaptomids have oviducal cycle lengths between 35 and 75 days at 4°C (Jersabek and 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 and Dam 2015). Although the typical astaxanthin content of spermatophores is not known, our recent results suggest that it might be coupled to fatty acid 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 during the reproduction period. Therefore, although astaxanthin precursors and  $\omega$ 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 primary productivity 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 follow seasonal dynamics that are closely similar in the copepods (Fig. II.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 fatty acids in both energy storage and structural lipids are well established (Brett and Müller-Navarra

1997; Arts 1999), while the functions of astaxanthin in eggs and/or nauplii are still subject to debate. Photoprotection is not needed in this boreal lake where UVR is attenuated to 1% or less of sub-surface radiation within the first meter (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 et al. 2013). Effective antioxidant protection may be particularly important in the non-feeding early naupliar stages, 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 and Gonsebatt 2012), thus increasing the requirement for antioxidant defences.

Although maximum egg abundance was much higher in spring as compared to summer, the numbers of eggs produced by each cohort were relatively similar. The reduction of both astaxanthin and  $\omega$ 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  $\omega$ 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, emphasizing the essential role of astaxanthin and fatty acids for copepod reproduction.

The magnitude of planktonic resource accumulation becomes apparent on the ecosystem level. During most of the ice-free period astaxanthin precursor carotenoids and  $\omega$ 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  $\omega$ 3 FAs (Fig. II.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  $\omega$ 3 FAs at rising temperatures (Hixson and 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 show 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 considerable resource stocks then enable a reproductive effort that could not be sustained by winter primary production alone.

## **References**

The references of each individual chapter have been included in the reference section at the end of the thesis.

## *Acknowledgments*

We thank Pierre Carrier-Corbeil and Marie-Josée Martineau for assistance in field and lab. This research was financed by the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, the Canada Foundation for Innovation, and the Fonds québécois de la recherche sur la nature et les technologies.

### III. EXPERIMENTAL TEST OF UVR AND TEMPERATURE EFFECTS ON COPEPOD PIGMENTATION

MANUSCRIPT

**Title:** Natural levels of ultraviolet radiation in a boreal brown-water lake do not affect copepod survival or photoprotective pigmentation

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Running head: UV and temperature effects on copepod carotenoids

Keywords: carotenoids, water temperature, zooplankton.

## **Abstract**

Survival of copepods is negatively affected by high ultraviolet radiation (UVR) and their carotenoid pigmentation has been described as a plastic adaptation to high levels of UVR. Water temperature further interacts with the UV-induced mortality and the need for photoprotective pigmentation as it modifies the efficiency of the enzymatic photorepair mechanism that alleviates photodamage. To study the confounding effects of UVR and temperature on zooplankton survival and pigmentation we exposed *Leptodiaptomus minutus* copepods to different irradiance conditions (darkness, visible light only, visible light and UVR) and temperature (5 ° C and 15 °C) in two laboratory experiments for a duration of up to 22 days. The experiments were performed on field-collected animals and were conducted in late winter and in early summer to investigate the responses of animals acclimated to the darkness under the ice versus those that had been exposed to UVR in open water. UVR levels were set to represent natural UVR exposure close to the lake surface at the time of each experiment. UVR exposure did not affect copepod survival or carotenoid content. Warmer temperature significantly reduced survival in both experiments and led to lower final carotenoid content, particularly in winter. We argue that this reduction in carotenoids was not due to reduced need of photoprotection. Rather it was linked to the copepod life cycle as indicated by different age structure of the copepod population in winter and summer and modified by ambient temperature. These results suggest that natural levels of UVR in a boreal brown-water lake do not affect copepod life span or carotenoid retention.

## **Introduction**

Organisms confronted with environmental change may adapt to a specific set of environmental conditions by means of phenotypic plasticity (Whitman and Agrawal 2009). One interesting such case is the carotenoid pigmentation of aquatic

microcrustaceans that constitute large sections of the plankton in both marine and freshwater ecosystems. Although usually not able to detect the pigment themselves due to their limited visual capabilities, strongly pigmented zooplankton are generally more susceptible to visual predators (Hairston 1979a; Gorokhova et al. 2013) hence they may reduce their carotenoid content when exposed to predator cues (Hansson 2004; Hylander et al. 2012). At the same time, numerous observations of pronounced intraspecific variability in pigmentation indicate that pigmentation must provide some advantage under certain conditions. In copepods, 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 as well as by experiment (Hairston 1976; Moeller et al. 2005; Hylander et al. 2009; Rautio and Tartarotti 2010; Sommaruga 2010). UVR decreases the survival of zooplankton (Rautio and Tartarotti 2010). Carotenoids may provide protection via the quenching of reactive oxygen species produced during UVR exposure (Krinsky 1979; Cockell and Knowland 1999). Carotenoids may also protect copepods from other sources of oxidative stress such as metal 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. Crustaceans cannot produce carotenoids *de novo*; instead they rely on the assimilation of dietary precursors that they may modify to obtain specific carotenoids (Matsuno 2001). The major carotenoid in copepods, astaxanthin, is typically derived from precursor carotenoids such as  $\beta,\beta$ -carotene, zeaxanthin and possibly lutein (Andersson et al. 2003; Rhodes 2006). Dietary availability of appropriate precursors is crucial for the copepods' ability to accumulate carotenoids (Andersson et al. 2003).

The effect of UVR on the survival of organisms is also linked to the presence of photorecovery radiation (PRR) and temperature. PRR, which consists of PAR and/or near-UV radiation (Williamson et al. 2001) is required in the light-dependent photoenzymatic repair (PER) (Hansson & Hylander 2009; Rautio & Tartarotti 2010).

The efficiency of this enzymatic process is affected by temperature, and the temperature-dependence of PER has been demonstrated experimentally in *Daphnia catawba* and *Leptodiaptomus minutus* (Williamson et al. 2002). In these experiments, animals were exposed to UV radiation (280–400 nm) at different temperatures (10–25 °C) in the presence vs. absence of PRR. Neither species survived the UVR exposure in absence of PRR. When PRR was present, both species displayed increased survival at higher temperatures. In *L. minutus*, survival was lowest at 15 °C, highest at 25 °C, and intermediate at 10 °C and 20 °C, respectively (Williamson et al. 2002). Thus, UV-exposed copepods might benefit from high carotenoid content at low water temperatures to counteract the reduced efficiency of enzymatic UVR repair (Williamson et al. 2002; Hansson and Hylander 2009).

However, not all data have convincingly shown that pigment accumulation is regulated by UVR. Observations of highly pigmented copepods in winter under the ice (Hairston 1979a; Hansson 2004; García et al. 2008) imply that photoprotection is not the only function of carotenoids in zooplankton. We have recently demonstrated that high winter pigmentation is related to the copepod reproductive cycle in a temperate lake (Schneider et al. 2016, 2017): copepods accumulate carotenoids along with storage lipids around the onset of winter and reduce both type of molecules towards the end of winter, reallocating these reserves towards eggs and, possibly, spermatophores. Thus there is a need for better understanding of the driving roles of temperature, UVR exposure and possible interactions with the copepod life cycle and time of year for the accumulation of carotenoids in copepods.

Our objective in this study was to test whether copepods would change their level of carotenoid content in response to (a) water temperature and/or (b) UVR exposure, and whether such a response would differ among (c) seasons. To do so, we experimentally examined how survival and carotenoid content in field-collected *Leptodiaptomus minutus* copepods respond to different solar radiation (UV,

UV+PAR, dark) and to temperature (5 and 15°C) at two times of year (late winter and early summer). In the experiment carried out in winter, the UV and PAR doses corresponded to the irradiance at ice-out. In the summer the doses were increased to correspond to the seasonally higher irradiance in the lake. To estimate the complementary effect of temperature-dependent enzymatic UV repair (PER) on copepod pigmentation, the experiments were run at low and high temperature (5 and 15°C). Assuming the carotenoids serve as photoprotective pigments, we expected their content in copepods to decrease in the absence of UVR. However, since the field-collected organisms contained high concentrations of carotenoids (Schneider et al. 2016), we expected little or no UV-induced mortality during the experiment. We also hypothesized that since the efficiency of PER is the better the higher the temperature, the copepods would require less photoprotective pigmentation with increasing temperature, thus would have a lower concentration of carotenoids in the warmer temperature treatment. In the absence of UV radiation, we expected to find no difference in pigmentation among different temperature treatments.

## **Methods**

### *Origin of study organisms*

The experiments were performed on *Leptodiaptomus minutus*, a common calanoid copepod widely distributed in Eastern North America. We used *L. minutus* from Lake Simoncouche, which is a mesotrophic lake situated in the Laurentides Wildlife Reserve in Quebec, Canada (48.23 °N, 71.25 °W; elevation 347 m a.m.s.l.). This dimictic lake covering an area of 87 ha is relatively shallow ( $Z_{\max} = 8$  m) and is entirely surrounded by boreal forest. 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

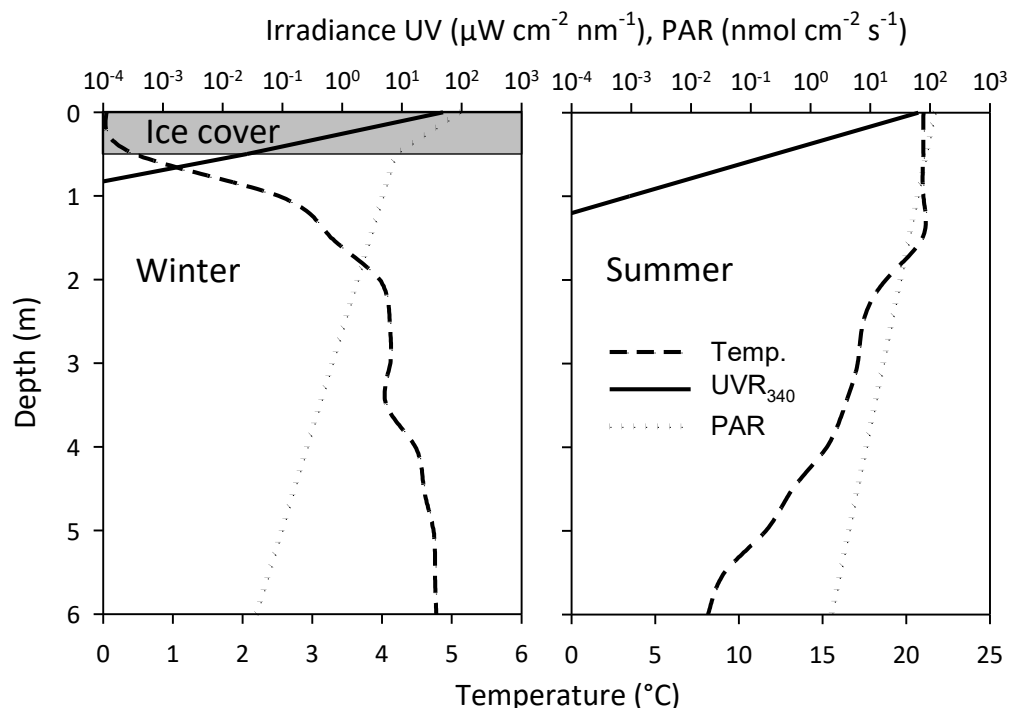


August. Dissolved organic carbon concentrations range between 4.1 and 8.3 mg C L<sup>-1</sup>.

UVR exposure in Lake Simoncouche has been assessed in 2011–2013 using a submersible profiler radiometer, the PUV-2500 (Biospherical Inc., San Diego, CA, USA). The high-resolution profile so obtained allowed for reliable calculation of the attenuation coefficients throughout the year, which were then used in combination with modeled surface UVR to estimate UV irradiance at a certain depth as in Schneider et al. (2016).

### *Experiment*

A 2 × 2 × 3 factorial laboratory experiment was performed in order to test whether *L. minutus* adapt their carotenoid content in response to radiation exposure and water temperature and whether this response would vary with season. Three conditions of radiation exposure were tested: darkness, visible light (here PAR) only, and PAR plus UVR. Temperature was set to 5°C and 15°C, respectively. The experiment was conducted with zooplankton sampled in March and in June to test the effect of different ambient conditions and life histories. The two temperature treatments corresponded to the mean water-column temperature in the lake in March and June, respectively (Fig. III.1). UVR exposure at 340 nm was set to simulate water-column irradiance at 0.4 m (Fig. III.1) at the top of the microcosms. Taking into account the height of the microcosms of 12 cm and assuming that UVR and light attenuation would be very similar than in the lake since filtered lake water was used in the experiment, the copepods were offered a physical refuge corresponding to 0.52 m lake depth. To account for the higher solar irradiance in June compared to March (Fig. III.1), the UVR irradiance and exposure time were increased in the second experiment (Table III.1). PAR irradiance was kept at 27.5 nmol cm<sup>-2</sup> s<sup>-1</sup> in both experiments.



**Figure III.1** Vertical profiles of water temperature and solar irradiance (UVR at 340 nm and PAR) at noon in Lake Simoncouche corresponding to the two experimental runs. In both experiments, UVR at 340 nm was adjusted to simulate natural solar irradiance at 0.4 m depth without the ice cover. The profiles have been obtained on 3 April 2012 (winter) and on 4 July 2012 (summer).

The experimental setup consisted of two temperature-controlled climate chambers (PGR15, Conviron, Winnipeg, MB, Canada) at  $5^{\circ}\text{C}$  and  $15^{\circ}\text{C}$ , respectively. Inside each chamber, PAR was provided by sixteen T8 white fluorescent lamps and UVR was provided by two UVA-340 fluorescent lamps (Q-Lab Corporation, Westlake, OH, U.S.A.). The lamps were adjusted to provide a natural day/night cycle and realistic UVR irradiance and dose for the two experiments, respectively (Table III.1).

Two boxes were constructed of dark blue corrugated plastic sheets (Coroplast Ltd, Granby, QC, Canada) providing a closed compartment for the five dark treatments and ten small ones for each replicate of the PAR and PAR+UVR treatments. PAR and UVR reached those treatments through round openings above the designated beakers. These openings were alternately covered with Aclar (Honeywell international, Morristown, NJ, U.S.A) and Courtgard (CP Films, Martinsville, VA, U.S.A.) foils to transmit or block incoming radiation at wavelengths <400 nm, respectively (Fischer et al. 2006).

**Table III.1** PAR and UVR characteristics during the experiment. Exposure times in hours per day. UV irradiance ( $\mu\text{W cm}^{-2} \text{ nm}^{-1}$ ) and daily dose ( $\text{mJ cm}^{-2} \text{ nm}^{-1}$ ) at the top of the mesocosms. PAR irradiance was  $27.5 \text{ nmol cm}^{-2} \text{ s}^{-1}$  in both runs.

Experiment	PAR exposure	UVR exposure	UV irradiance	Daily UV dose
Winter	8	4	1	14.4
Summer	16	8	2.5	72

Individuals of *L. minutus* were collected by vertical net tows (50  $\mu\text{m}$ ) in Lake Simoncouche prior to each experiment. This initial population consisted solely of adults in winter, but mainly (>90 %) of CV-stage copepodites in summer, following the natural population structure in the lake (Schneider et al. 2017). The copepods were sedated with  $\text{CO}_2$  and carefully transferred to 500 mL plastic containers (100 individuals each, 30 replicates) containing 50  $\mu\text{m}$ -sieved lake water. The animals were subsequently transferred into the experimental microcosms (1 L glass beakers filled with GF/F-filtered lake water) and placed into the experimental boxes. The copepods showed active swimming behavior after this transfer. They were fed cultured *Scenedesmus* sp. cells every second day ( $18000 \text{ cells mL}^{-1} \text{ d}^{-1}$ ). Twice a

week, the water was replaced with fresh filtered lake water and the beakers were moved in rotation to a different position within the same treatment to equilibrate potential variations in exposure, for example due to irregularities in lamp output. In order to retain sufficient amounts of material for carotenoid analysis, the experiments were terminated when the survivor count in three or more microcosms had dropped below 5% as estimated during water replacement. The resulting run time was 22 days in winter and 18 days in summer. The surviving copepods in each beaker were counted and transferred into 1.5 mL-plastic tubes.

#### *Carotenoid quantification*

Carotenoids in copepods were extracted from lyophilized animals in ethanol 95% (v/v) as in Schneider et al. (2012). The extract was scanned from 300 nm to 850 nm in a Cary 100 Bio UV-Vis spectrophotometer (Varian Inc., Walnut Creek, CA, U.S.A.) and the carotenoid concentration was calculated from the corrected (i.e., zeroed at 750 nm) absorbance at 474 nm according to Hessen and Sørensen (1990) and expressed as ng carotenoids per individual. Carotenoids were normalized to the number of individuals rather than to dry mass because the latter could not be reliably determined for replicates with a low number of survivors.

#### *Data analysis*

Three-way analysis of variance (ANOVA) and post-hoc pairwise comparisons were applied to test whether season, temperature, light treatment or their interaction had a significant influence on copepod survival and carotenoid content. Normality and homogeneity of variance were assessed based on visual examination of the residuals, and square root of survival was used to meet the ANOVA assumptions. All tests were carried out using the software package JMP 10 (SAS Institute Inc., Cary, NC, U.S.A.).

## Results

Copepod survival differed considerably among treatments ranging from  $4.2 \pm 4.0$  % (winter, 15°C, dark) to  $85.8 \pm 10.3$  % (summer, 5°C, dark; Fig. III.2a). Survival was significantly influenced by all three factors (season, temperature and light) as well as by their interactions (ANOVA, Table III.2). However, post-hoc pairwise comparisons reveal that temperature was the main factor shaping survival in both the winter and the summer experiment, while the light treatment had little effect except for the higher survival in the dark treatments in summer (Fig. III.2a). Specifically, survival was not altered by the absence or presence of UVR as compared to visible light only (Fig. III.2a). In the summer experiment, the development of copepods was strongly affected by temperature, with only  $24 \pm 9$  % of the surviving population being adults in the 5°C treatment compared to  $89 \pm 12$  % at 15°C.

**Table III.2** ANOVA results showing factors explaining copepod survival at the end of the two experiments.

Source	df	F Ratio	P
Season	1	33.99	<.0001*
Temp	1	267.21	<.0001*
Season*Temp	1	24.93	<.0001*
Light	2	42.61	<.0001*
Season*Light	2	55.37	<.0001*
Temp*Light	2	5.53	0.007*
Season*Temp*Light	2	3.30	0.045*
Residual	48		

During both experiments, copepods reduced their carotenoid content in all treatments as compared to their initial carotenoid content of  $15.8 \pm 0.6 \text{ ng ind}^{-1}$  in winter and  $16.6 \pm 1.5 \text{ ng ind}^{-1}$  in summer (Fig. III.2b). The final carotenoid content varied between  $1.2 \pm 1.2 \text{ ng ind}^{-1}$  (winter,  $15^\circ\text{C}$ , dark) and  $12.5 \pm 1.1 \text{ ng ind}^{-1}$  (summer,  $5^\circ\text{C}$ , visible light + UVR). Similarly to survival, carotenoid content was significantly influenced by all three factors tested, but with fewer interactions involving the factor 'light' (Table III.3). Pairwise comparisons indicate that higher carotenoid content was retained in the summer experiment and that the effect of temperature was more pronounced in the winter treatment, while light had no effect except for the contrast between dark and visible only treatments in summer at  $15^\circ\text{C}$  (Fig. III.2b).

**Table III.3** ANOVA results showing factors explaining copepod carotenoid content at the end of the two experiments.

Source	df	F Ratio	P
Season	1	178.46	<.0001*
Temp	1	52.46	<.0001*
Season*Temp	1	5.55	0.023*
Light	2	12.93	<.0001*
Season*Light	2	2.86	0.068
Temp*Light	2	0.51	0.606
Season*Temp*Light	2	3.56	0.037*
Residual	43		

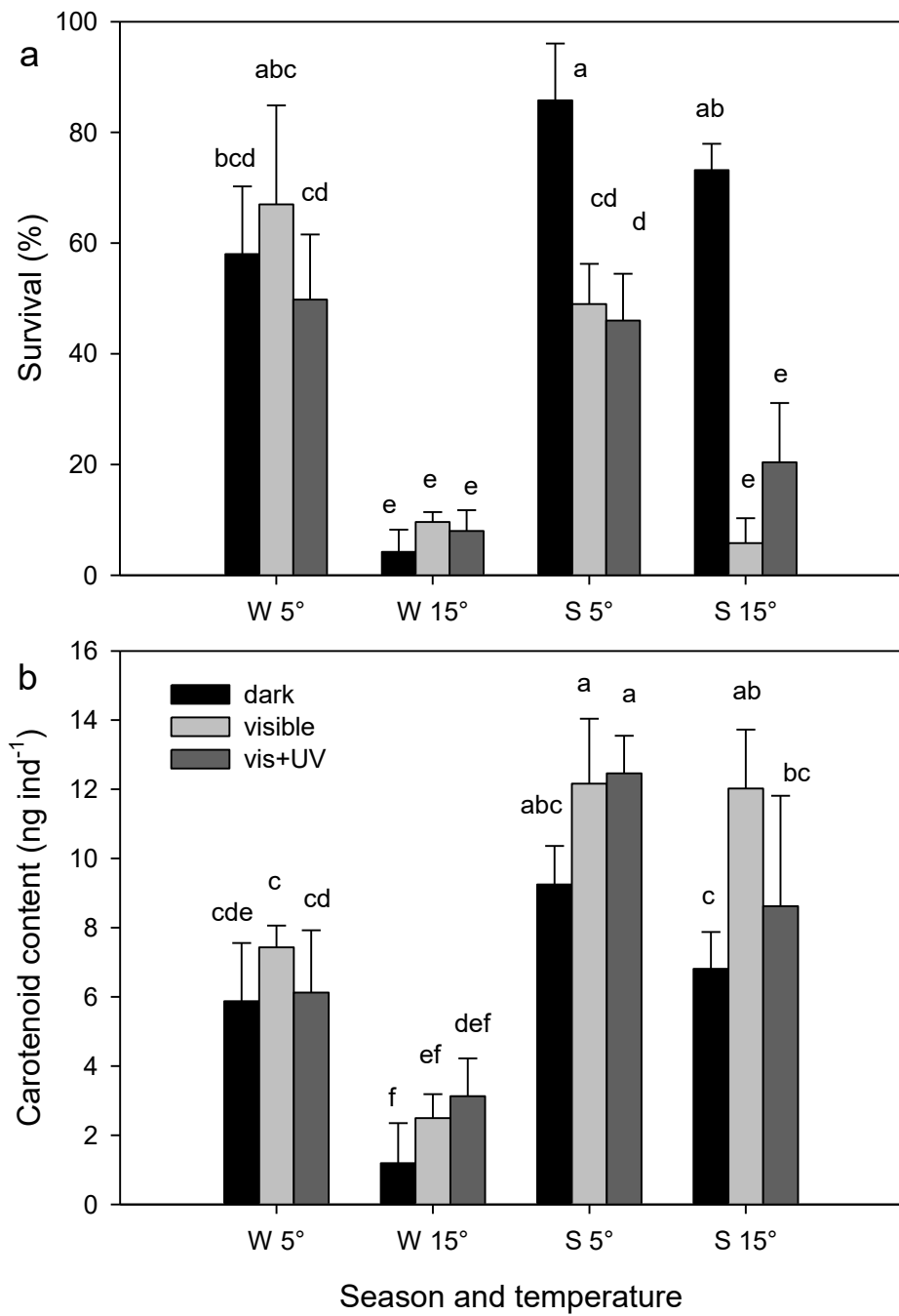


Figure III.2 (caption on following page)

**Figure III.2** Results of the two laboratory experiments showing (a) survival and (b) carotenoid content at the end of each treatment in winter and summer. The initial carotenoid content was  $15.8 \pm 0.6$  ng ind<sup>-1</sup> in winter and  $16.6 \pm 1.5$  ng ind<sup>-1</sup> in summer. W, winter; S, summer; 5°C and 15°C, temperature treatments. Legend for light conditions in panel (b); vis+UV, visible light plus ultraviolet radiation. Error bars represent the standard deviation.

The cultured *Scenedesmus* cells contained  $0.29 \mu\text{g mg}^{-1}$  of the astaxanthin precursors zeaxanthin and beta-carotene. Furthermore they contained  $2.28 \mu\text{g mg}^{-1}$  of lutein, which has been proposed as a potential precursor of astaxanthin in copepods. The regular addition of 18000 *Scenedesmus* cells mL<sup>-1</sup> d<sup>-1</sup> to the experimental incubations corresponded to daily ratios per copepod of: 15.5 ng lutein, 0.4 ng zeaxanthin, and 1.6 ng  $\beta,\beta$ -carotene. This was more than the highest measured daily increase in copepod astaxanthin,  $0.45 \text{ ng ind}^{-1} \text{ d}^{-1}$  (Schneider et al. 2016). The culture contained  $16.0 \mu\text{g mg}^{-1}$  of total FAME consisting of 34%  $\omega 3$  and 13%  $\omega 6$  FAs ( $\omega 3:\omega 6$  ratio of 2.5) dominated by C18 PUFAs, while longer-chain PUFAs such as ARA, EPA and DHA were absent.

## Discussion

The experiments showed that natural UVR irradiance and doses did neither affect survival nor carotenoid retention in *L. minutus* copepods. The UVR exposure in the experiment corresponded to natural levels of UVR at about 40–50 cm below the lake surface providing a realistic set-up for the observation of medium-term changes in the physiology of the organisms rather than damaging them with higher-than-natural UVR. Previous laboratory experiments have often been carried out applying relatively high UV irradiance, sometimes including UV-B radiation and focusing on



UV-induced damage (Williamson et al. 2002; Hansson et al. 2007). While useful to show the presence of phenotypic adaptation in principal, these experiments might not correctly simulate environmental and/or physiological drivers in low-UVR systems such as temperate brown-water lakes.

In the present study, exposure to UVR did neither affect copepod survival nor carotenoid content as compared to visible light only treatments. However, the absence of visible light increased survival during the summer experiment. The lack of effect from UVR on the carotenoid content of *L. minutus* of the same population has recently been demonstrated in a seasonal study in Lake Simoncouche (Schneider et al. 2016). These results are also in concordance with other recent observations that point towards non-photoprotective functions of carotenoids in copepods (Sommer et al. 2006; Schneider et al. 2012). In these studies, highly pigmented copepods have been reported in relatively UVR-opaque environments (the Baltic Sea and extremely turbid shallow lakes, respectively) suggesting that UVR exposure is not driving carotenoid accumulation in these systems. We propose that below a yet to be determined threshold of UVR exposure, carotenoid pigmentation is better explained by factors unrelated to UVR.

In both experimental runs copepods retained significantly less astaxanthin at 15 °C as compared to 5 °C. Our initial hypothesis that copepod carotenoid concentration would be negatively related to water temperature was assuming a primarily photoprotective role of the pigments. The rationale behind this idea was that the enhanced efficiency of enzymatic processes such as photo-repair at higher temperatures would reduce the need for non-enzymatic antioxidants such as carotenoids (Williamson et al. 2002; Häder et al. 2015). However, we found no indication that the ambient levels of UVR provided affected copepod survival or carotenoid content. Thus, replacement of enzymatic photoprotection by carotenoids does not seem plausible.

Although the experiment suggests a strong influence of temperature on *L. minutus* carotenoid content, these results should be interpreted with caution, as the drastically reduced survival rates in higher temperature treatments indicate largely differing physiological conditions in the respective populations. Copepods might have completed their life cycle faster (including ageing processes and death) in the warmer environment, as was indicated by the dominance of adults in 15°C treatments in summer. If the differences in survival reflect differential metabolic activity, this would mean that the copepod carotenoids were subjected to different physiological environments, potentially affecting their uptake, retention and utilization. Alternatively, water temperature may act as a physiological trigger to reduce pigmentation, either directly or indirectly via the process of reproduction. No egg sacs have been observed in either experiment, but they may have been lost in the course of the regular water exchange, as the mesh used was wide enough to let pass copepod eggs.

A general negative effect of temperature on copepod survival was found in both summer and winter. However, carotenoid retention was generally higher in summer as compared to winter, possibly due to the life history of the animals. At the end of winter, copepods were approaching the end of their lifespan with only about 40 days left until the spring egg peak (Schneider et al. 2017). In contrast, the copepods in the summer experiments were mostly juveniles that still had 72 days to go to their reproduction peak. These differences in relative age were likely further increased by the temperature treatments, resulting in accelerated development of the winter population at 15°C, while the development of the summer generation was slowed down at 5°C. The latter was also indicated by the large proportion of juvenile specimen at 5°C as compared to predominately adults at 15°C. Thus, the summer generation was programmed to accumulate resources at the time of the experiment, while the winter generation was scheduled to reduce them, partly due to investment into reproduction (Schneider et al. 2017).

*Scenedesmus* has been widely used as a food source in copepod experiments (Brüsin et al. 2016), albeit sometimes combined with other algae (Hansson et al. 2007). The algae have previously deemed appropriate diet for carotenoid accumulation experiments due to their high content of astaxanthin precursors (Brüsin et al. 2016). HPLC analysis of the *Scenedesmus* culture confirmed that the most dominant carotenoid in this species is lutein, which has been proposed as a potential precursor of astaxanthin in copepods (Rhodes 2006). Furthermore, the generally accepted astaxanthin precursors, zeaxanthin and  $\beta,\beta$ -carotene, were available in sufficient quantities to allow astaxanthin accumulation at the maximum rate determined in this population (Schneider et al. 2016). *Scenedesmus* contained a high amount of PUFA, and 34% of total FAs were  $\omega$ 3 FAs. However, they lacked some FAs considered essential, in particular DHA, which is typically present in copepods in high concentrations. We have recently documented that *L. minutus* co-accumulate astaxanthin together with fatty acid reserves but independently from UVR exposure (Schneider et al. 2016). Thus, it cannot be excluded that failure to accumulate astaxanthin during the experiment was partly due to the lack of appropriate dietary fatty acids. Nevertheless, this would imply that UVR exposure alone did not suffice to trigger astaxanthin accumulation and thus would not contradict our principal conclusion. An alternative explanation why copepods reduced carotenoid content may be the perceived threat by visual predators (Hylander et al. 2012). This threat is communicated to the copepods via kairomones, i.e., chemical cues indicative of fish presence that were also potentially present during the experiment via the regular addition of filtered lake water.

Together, the experimental results confirm the view proposed in our earlier study that natural levels of UVR in a temperate brown-water lake are insufficient to damage copepods or induce carotenoid accumulation (Schneider et al. 2016). Water temperature may play a key role as a controlling factor, either directly as an environmental cue or indirectly by affecting copepod development and metabolic

activity, subsequently leading to a readjustment of the astaxanthin content. The results emphasize that, in addition to photoprotection, other functions of carotenoids also need to be considered in order to obtain a complete understanding of the ecological driving forces and adaptive significance of these pigments in zooplankton, particularly in low-UVR systems.

## References

The references of each individual chapter have been included in the reference section at the end of the thesis.

## *Acknowledgments*

We thank David-Alexandre Gauthier, Aurélie Guillou, Pierre Carrier-Corbeil and Denis Walsh for field and laboratory assistance, Craig E. Williamson for providing the UV filter foils, and Warwick F. Vincent and Marie-Josée Martineau for HPLC pigment analysis. This research was financed by the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, the Canada Foundation for Innovation, and the Fonds québécois de la recherche sur la nature et les technologies.

## Supplementary material

The suitability of cultured *Scenedesmus* as a food source during the experiment was evaluated based on their content in precursor carotenoids for astaxanthin and in fatty acids, which were analyzed as follows. A sample of the *Scenedesmus* culture was obtained after the experimental runs to evaluate the dietary quality of the algae. The culture was filtered on GF/F glass fiber filters for analysis of pigments and fatty acids

(three replicates each). The filters for pigment analysis were kept frozen at  $-80^{\circ}\text{C}$  until analysis via HPLC as in Schneider et al. (2016).

The filters for fatty acid analysis had been pre-weighed before filtration and then kept at  $-80^{\circ}\text{C}$ . The filters were freeze-dried and their weight determined prior to extraction and subsequent analysis via gas chromatography.

#### *Scenedesmus carotenoids*

Carotenoid pigments in *Scenedesmus* were analyzed by reversed-phase high-performance liquid chromatography (HPLC). The frozen seston filters were extracted by rod sonication (Microson XL2000, Misonix, Farmingdale, NY; three times 20 s on ice at 10 W) in 95% (v/v) aqueous methanol. The extracts were incubated for 30 min on ice under argon atmosphere, then centrifuged and filtrated through  $0.2\ \mu\text{m}$  polytetrafluorethylene membrane filters (VWR international, Mississauga, Ontario, Canada) and stored at  $4^{\circ}\text{C}$  in the dark under argon gas until HPLC analysis within 48 h.  $50\ \mu\text{L}$  were injected into an Accela 600 HPLC system (Thermo Scientific, Waltham, MA, U.S.A.) equipped with a Hypersil Gold C8 column ( $150\ \text{mm} \times 4.6\ \text{mm}$ ,  $3\ \mu\text{m}$  particle size, Thermo Scientific) protected by a Hypersil Gold C8 guard column ( $10\ \text{mm} \times 4\ \text{mm}$ ,  $3\ \mu\text{m}$  particle size, Thermo Scientific) using the HPLC protocol of Zapata et al. (2000). The run-time was 40 min. 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 quantified based on the absorbance chromatogram at 450 nm (Bonilla et al. 2005).

#### *Scenedesmus fatty acids*

Lipids from freeze-dried filters were extracted and transmethylated in methanol : toluene : acetyl chloride mixture (4000 : 1000 : 125) at  $90^{\circ}\text{C}$  for 20 min; the resulting

fatty acid methyl esters (FAMES) were separated from non-FAME components by addition of water 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, CA) 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  $\mu\text{m}$  film thickness). Helium was used as the carrier gas (flow rate 1  $\text{mL min}^{-1}$  with electronic pressure control) and the temperature ramp was as follows: 70°C for 1.5 min followed by an increase of 20°C  $\text{min}^{-1}$  until 110°C, an increase of 12.5°C  $\text{min}^{-1}$  until 160°C, and an increase of 2.5°C  $\text{min}^{-1}$  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) versus an internal standard (nonadecanoic acid) using calibration curves based on known standard concentrations.

#### *Scenedesmus availability to copepods*

The availability of dietary resources to copepods during the experimental incubations was calculated based on the cellular content of cultured *Scenedesmus* in carotenoids and fatty acids combined with the daily addition of cultured cells to the treatments ( $1.8 \times 10^5$  cells copepod $^{-1}$  d $^{-1}$ ). The requirement of astaxanthin precursors in copepods was estimated as the maximum astaxanthin accumulation rate in the wild population (Schneider et al. 2016), which was converted from  $\mu\text{g mg}^{-1}$  d $^{-1}$  to ng ind $^{-1}$  d $^{-1}$  using copepod dry mass.

## GENERAL CONCLUSIONS

### **Seasonal variation**

Lake Simoncouche is characterized by pronounced seasonal variation in its abiotic and biotic environment. For instance, water temperature, exposure to UVR, and phytoplankton abundance all differed markedly between the open water seasons versus the ice-covered period (Fig. I.2a, b).

The total carotenoid concentration in *L. minutus* in Lake Simoncouche was within the range previously described for this species (Moeller et al. 2005; Rautio et al. 2009). Carotenoid content changed considerably 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. For the duration of winter, the water column was characterized by highly pigmented copepods whose elevated astaxanthin concentration was related to the high concentration of lipids in the copepods and to low predation pressure (Fig. I.1a).

This variation was also reflected in the rate of change, *i.e.*, the increase and reduction of copepod carotenoid content, on both the individual and the population level (Chapters I and II). Thus, net accumulation of carotenoids was restricted to the period from October to early January, while during the remaining months carotenoid content remained stagnant or was reduced (Fig. I.1b). On the ecosystem level, the allocation of astaxanthin and its precursor carotenoids switched from seston to copepods during winter (Fig. II.3).

### **Drivers of copepod carotenoid accumulation**

The results of multiple linear regression analysis provide evidence of a strong link between copepod astaxanthin and lipid content as well as investment into reproduction (Chapter I). These two explaining factors are complemented by additional effects of temperature, diet availability and predation by young-of-the-year fish.

The seasonal pattern of copepod pigmentation in Lake Simoncouche cannot be explained by photoprotection. If exposure to UVR was a major driver of carotenoid pigmentation, copepods would be expected to increase their pigment concentration according to UVR exposure. However, the rate of astaxanthin accumulation was statistically unrelated to underwater UV irradiance, and absolute astaxanthin concentration was even inversely correlated with UVR (Table I.1). In concordance with these field observations, the experiments showed that natural levels of UVR irradiance and doses did not affect accumulation and loss rates of astaxanthin in this copepod (Fig. III.2).

Copepod fatty acid and carotenoid content both reached their maximum values in winter and had a clear minimum in September-October (Chapter I). The esterified fractions of copepod astaxanthin content were correlated with total fatty acid content, while free astaxanthin was slightly negatively correlated, suggesting a physiological connection between lipid reserves and astaxanthin esters.

We observed two distinct periods of net loss of astaxanthin in late winter and in late summer, coinciding with the peaks in the egg carrying ratio of the winter and summer generations, respectively. Accordingly, net accumulation of astaxanthin only occurred when the egg ratio was low, showing that astaxanthin accumulation and egg production were temporally separated and suggesting that the build-up of astaxanthin



reserves was counteracted by investment into reproduction (Chapter I). 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 reduction 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 et al. 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 et al. 2004).

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 temporal changes in copepod carotenoid content have been linked to seasonal shifts in predation pressure relative to UVR exposure (Hansson 2004). The absolute astaxanthin concentrations in the present study were significantly negatively related to the YOY fish predation, consistent with such a response, and predation was also among the top variables selected by the multiple linear regression 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.

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,  $\beta,\beta$ -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 pre-winter 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 (Chapter I). 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 non-enzymatic 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.

Although the experiment (Chapter III) suggests a strong influence of temperature on *L. minutus* pigmentation, these results should be interpreted with caution, as the drastically reduced survival rates in higher temperature treatments indicate largely differing physiological conditions in the respective populations. Copepods might have completed their life cycle faster (including ageing processes and death) in the warmer environment, as was indicated by the dominance of adults in 15°C treatments in summer. If the differences in survival reflect differential metabolic activity, this would mean that the copepod carotenoids were subjected to different physiological environments, potentially affecting their uptake, retention and utilization.

### **Transfer of carotenoids and fatty acids from phytoplankton to copepod eggs**

In Chapter II we show that in our study system copepod reproduction at the end of winter would be strongly limited by the available dietary resources. We argue that under these conditions the build-up of reserves provides a solution allowing the copepods to reproduce at the optimal time of the year via capital breeding. Indeed, the periods where copepods reduced their astaxanthin and fatty acid content overlapped with the increase in egg production in late winter and in summer. Complementarily, the overall high abundance of phytoplankton pigments in late fall favored astaxanthin accumulation (Fig. II.2). This period in November and December appears to be particularly crucial for the accumulation of astaxanthin and FA reserves. Astaxanthin and FAs are both transferred to eggs and follow very similar seasonal dynamics in

copepods, 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 magnitude of planktonic resource accumulation becomes apparent on the ecosystem level. During most of the ice-free period astaxanthin precursor carotenoids and  $\omega$ 3 FAs are more abundant in the lake seston than in copepods, but during winter this relationship is reversed and copepods become the major storage of the derived carotenoid astaxanthin and of  $\omega$ 3 FAs (Fig. II.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  $\omega$ 3 FAs at rising temperatures (Hixson and Arts 2016), this might result in limited availability of high-quality lipids for aquatic secondary consumers.

### **Implications of the results**

This study is among the first to test all of the major competing hypotheses aiming to explain zooplankton pigmentation in a natural ecosystem after Nelson G. Hairston Jr.'s pioneering work relating the expression of carotenoids in copepods to photoprotection and visual predation (Hairston 1976, 1979a).

Carotenoid accumulation in copepods has been presented as a plastic trait whose expression results from the relative importance of two external threats: exposure to UVR and visual predation. One major contribution of this thesis is the finding that UVR exposure does not explain the seasonal changes in copepod carotenoid content, leading to the subsequent conclusion that photoprotection is not the main function of

carotenoids in this copepod (Chapters I and III). These results align with an increasing number of publications including my own previous work that conclude that UVR exposure is not always a driving factor of carotenoid accumulation in copepods (Sommer et al. 2006; Schneider et al. 2012; Hylander et al. 2015).

Chapter I provides the first comprehensive evidence linking changes in copepod pigmentation to their fatty acid content through the seasonal dynamics of free versus esterified astaxanthin between the poles of reserve building and investment into reproduction. Chapter II is the first study to estimate production and transfer rates of astaxanthin and essential fatty acids in a natural copepod population. Furthermore, we show that during several months of the year, the amount of carotenoids and fatty acids accumulated in zooplankton supersedes the respective concentration in the lake seston. These results underline the central role zooplankton plays within the lake ecosystem, particularly during the under-studied winter period.

### **Potential future research directions**

In my doctoral project I have comprehensively studied a population of copepods in one lake. The next step of research on the subject should aim at securing and extending this knowledge by providing replication within the boreal biome based on similar lakes as well as by adding observations of other species in a more diverse range of systems showing strong seasonality, such as polar and alpine lakes.

We have demonstrated that astaxanthin accumulation is linked to the build-up of lipid reserves and that carotenoids together with fatty acids are passed on to copepod offspring during reproduction. Our methodological approach offered an ecological perspective on the roles and functions of astaxanthin in a temperate lake. However, we did not investigate in detail the physiological interactions between carotenoids and fatty acids and their role in copepod metabolism, performance and development.

Possible research questions could be: What is the function of astaxanthin esters in copepod lipid storage, eggs and nauplii on the cellular level? How are these biochemical interactions related to the fatty acids the carotenoids are esterified with? Do these functions differ from those of free or protein-bound astaxanthin? Some studies suggest that normal cellular processes might generate enough oxidative stress to damage the cell and that astaxanthin might improve performance of an organism by keeping oxidative stress at a minimum (Łotocka et al. 2004; Gorokhova et al. 2013). It will be very interesting to look further into the variety of physiological processes that involve astaxanthin in its different forms.

One logistical challenge that we were not always able to solve was the difficulty of sampling around the time of ice formation and of ice-off, respectively. During these periods the lake is predominantly ice-covered, making it impossible to sample by boat while the ice is not robust enough to allow for safely entering it by snowmobile carrying the heavy equipment. This is particularly regrettable as these periods are of high limnological interest for better understanding the transition between the two opposite seasonal regimes that define the conditions affecting so many biological processes in the lake. Appropriate technical equipment may at some point allow for a more complete coverage of these critical moments in the year.

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