Exceptional lipid storage mode of the copepod *Boeckella poopoensis* in a pampean salt lake, Argentina

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ABSTRACT: The lipid biochemistry of zooplankton was investigated in Lake Chasico, a pampean salt lake in Argentina. The lipid biomass of the zooplankton community was dominated by the calanoid copepod *Boeckella poopoensis*. The major storage lipids during winter were wax esters and triacylglycerols, which reached up to 59 and 37 % of the total lipids, respectively. A striking feature of the zooplankton fatty acid composition was the extraordinarily high level of 18:4(n-3) and 20:4(n-3) fatty acids, the highest ever reported for the latter in zooplankton. During winter, 20:4(n-3) accounted on average for 20 % of the total fatty acids in the wax ester fraction and 7 % in the triacylglycerols. The close relationship (r = 0.83, p < 0.001) between the 2 fatty acids implies the biosynthesis of 20:4(n-3) in *B. poopoensis* by chain elongation of 18:4(n-3), a dietary precursor and flagellate marker. The accumulation of 20:4(n-3) may be also partially related to *B. poopoensis* grazing on heterotrophic protozoa or non-flagellated chlorophytes, although this fatty acid was almost absent in the seston fraction. In summer, wax esters were slightly lower (45 %), compensated by higher phospholipid levels. The 16:0 fatty alcohol moiety was predominant in the wax esters of all samples, corroborating the opportunistic feeding behavior of *B. poopoensis*. The high amounts of wax esters in zooplankton are typical of marine species, suggesting that the wax ester biosynthesis of *B. poopoensis* and the extraordinary fatty acid composition are adaptations to the unstable environmental conditions of salt lakes.

KEY WORDS: Polyunsaturated fatty acids · Biosynthesis · Trophic markers · Wax esters · 20:4(n-3)

INTRODUCTION

Many zooplankton species are known to store large amounts of lipids. These storage lipids play an important role during reproduction, food scarcity, ontogeny and diapause. Copepods, in particular, are able to accumulate copious amounts of long- and short-term reserves of lipids, such as wax esters and/or triacylglycerols. The precise functional significance of the different types of lipids stored and the different chain lengths of their moieties, fatty acids and fatty alcohols is not yet clear, but they probably reflect specific life-cycle strategies of adaptation to different environments (Lee et al. 2006, Kattner & Hagen 2009).

Some fatty acids are important trophic markers because they are incorporated unchanged from the diet into consumers, providing information on predator–prey relations (Dalsgaard et al. 2003). Moreover, polyunsaturated fatty acids are important compounds that can limit the somatic growth and reproduction of zooplankton (Müller-Navarra et al. 2000, Ravet et al. 2003). These fatty acids, particu-

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larly 22:6(n-3), 20:5(n-3) and 20:4(n-6), are essential for maintaining the structure and functions of cell membranes and are precursors of eicosanoids. They are mainly derived from phytoplankton and are vital for higher trophic organisms.

How far zooplankton species are able to elongate or desaturate dietary fatty acids is still under discussion. Bell et al. (2007) found experimental evidence that some marine species were unable to synthesize polyunsaturated fatty acids at ecologically significant rates. Conversely, freshwater zooplankton are believed to retain 18:3(n-3) and 18:2(n-6) and convert them into polyunsaturated fatty acids (Bec et al. 2003a), and harpacticoid copepods can actively synthesize essential fatty acids in significant quantities (Nanton & Castell 1999). Protozoans seem to convert or 'upgrade' dietary fatty acids to longer chain polyunsaturated fatty acids (Klein-Breteler et al. 1999, Bec et al. 2010). However, zooplankton species are usually able to cover their polyunsaturated fatty acid demands from their food.

The lipid composition of zooplankton and its biological implications have mostly been studied in marine species, particularly in polar regions (reviewed by Lee et al. 2006, Falk-Petersen et al. 2009, Kattner & Hagen 2009). Less is known about freshwater copepods (Brett et al. 2009, Burns et al. 2011) and nothing at all is known about the lipids and fatty acids in zooplankton in South American salt lakes. The calanoid copepod *Boeckella* spp. is an important species that frequently occurs in the Sub-Antarctic, central Chilean, Patagonian and Paramo-Puna regions (Menu-Marque et al. 2000). The species *Boeckella poopoensis* is a typical copepod of salt lakes and has also occasionally been found in marine waters (Hoffmeyer 1983).

The present study aims at a better understanding of the lipid composition and storage mode of *B. poopoensis*, which, like marine calanoid copepods, has a high accumulation of wax esters but differs from freshwater species. We hypothesize that the salty environment of Lake Chasicó, Argentina is responsible, or at least stimulates, the accumulation of lipids in *B. poopoensis.*

MATERIALS AND METHODS

A survey of zooplankton and seston lipid biochemistry was carried out in Lake Chasicó. This shallow saline lake (maximum depth 16 m, salinity ~20) is a nature reserve located in the semiarid region of the Argentinean Pampa. The winter samples were collected monthly in July, August and September 2007 and 2008, and summer samples were collected in January, February and March 2008. The sampling sites (Fig. 1) were Station CV, near Chapalcó Village (6 m depth), Station EE, near the place called 'El Embudo' (3 m), and Station EV, near 'El Vivero' close to the River Chasicó mouth (2.5 m). Zooplankton was collected during daytime at each sampling site by 20 vertical net tows (200 µm mesh size) from the lake bed to the surface. The filtered volume was calculated with a digital flowmeter with back-run stop (Hydrobios). Lake water was sampled at 1 m depth with a Van Dorn sampler.

Live zooplankton were transferred into 500 ml of filtered lake water and mixed homogenously by bubbling air through the sample with a small pump. From this single pooled bath, 2 aliquots of 100 ml each were filtered through glass fiber filters (Whatman GF/F, pre-combusted at 500°C for 5 h) containing roughly 200 specimens. One filter was preserved in dichloromethane:methanol (2:1 by volume) under nitrogen atmosphere at −30°C, and the other was dried overnight (60°C), weighed and stored in a desiccator. Additional net samples were collected as described above and preserved with buffered formaldehyde (4%), and the number of specimens was counted in a zooplankton counting chamber (Hydro-Bios) under a stereoscopic microscope. For seston samples, lake water (0.5 to 1 l) was filtered through pre-combusted glass fiber filters (zooplankton was removed before filtration). The filters were preserved dried or in dichloromethane:methanol, as described above.
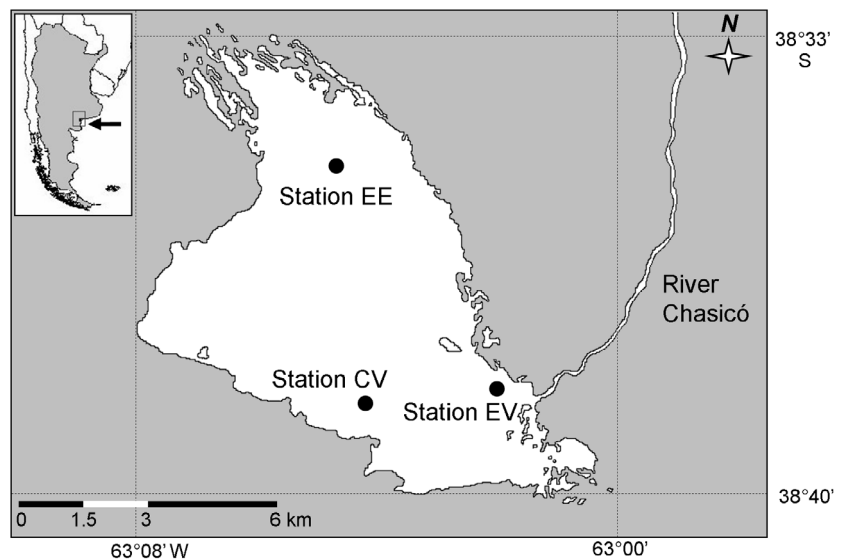

Fig. 1. Location of Lake Chasicó and sampling stations

For the determination of particulate organic carbon (POC), the dried filters were homogenized with a ball mill (Retsch PM 100) and analyzed by high temperature flash combustion using an elemental analyzer (Carlo Erba NA 2100). The samples in dichloromethane:methanol solution were homogenized with a high performance disperser (Ultra-turrax), and lipid extraction was performed basically after Folch et al. (1957); this protocol was slightly modified by using the less hazardous dichloromethane, which has a similar extraction efficiency as chloroform. After gravimetrical determination of total lipids, the lipid extracts were transesterified under nitrogen atmosphere with 3% concentrated sulfuric acid in methanol for 4 h at 80°C. The resulting fatty acid methyl esters and free fatty alcohols were extracted with hexane and simultaneously analyzed using gas-liquid chromatography (Hewlett Packard 6890 GC) on a 30 m wall-coated capillary column (i.d. 0.25 mm, film thickness 0.25 μm; liquid phase DB-FFAP) using temperature programming according to Kattner & Fricke (1986). Fatty acids and alcohols were quantified with an internal 19:0 fatty acid methyl ester standard and identified with standard mixtures. Confirmation by GC-MS was carried out where necessary.

Lipid classes were determined from samples of the 3 sampling sites representative for winter (August 2007 and 2008) and summer (February 2008). The lipid classes were separated by HPLC (LaChromElite HPLC system) after Graeve & Janssen (2009). Due to the high amount of the 20:4(n-3) fatty acid in the winter zooplankton, the lipid samples from August were separated by HPLC, and, after transesterification, the fatty acid and alcohol compositions of the neutral lipid classes were analyzed as described above for total lipids.

RESULTS AND DISCUSSION

The calanoid copepod *Boeckella poopensis* was always present in the zooplankton samples (Table 1), reached its maximum abundance in summer and predominated exclusively in the winter of 2007. The rotifer *Brachionus plicatilis* was generally more abundant in the summer and winter of 2008, and the cladoceran *Moina eugeniae* was only present in the summer of 2008. Owing to the small size or biomass of the lipid-poor *M. eugeniae* and *B. plicatilis*, the lipid composition

of all samples was determined mainly by the large and lipid-rich *B. poopoensis* specimens. As calculated from the pure *B. poopoensis* sample, the lipid content (% organic carbon) was ~40% in winter 2007, placing this copepod in the group of zooplankton species with moderate lipid contents (Kattner & Hagen 2009).

Wax esters were dominant during the winter of 2007, with on average 59% of total lipids, whereas in winter and summer of 2008, they constituted 47 and 45%. Triacylglycerol (39%) and polar lipid (13%) levels were slightly higher in summer (Table 1). The wax ester content of *Boeckella poopoensis* was lower than that of wax ester-rich marine copepods, such as polar *Calanus* spp., which accumulates up to 90% of total lipid as wax esters (Lee et al. 2006). The main fatty alcohol moiety of the wax esters was 16:0 (Tables 2 & 3), accounting for >80% of total fatty alcohols in both seasons. Other fatty alcohols were 14:0 and the 2 isomers 18:1(n-7) and (n-9). The 16:0 alcohol predominates also in *Clausocalanus* spp. from the Red Sea (Cornils et al. 2007). In polar zooplankton species, 16:0 and 14:0 were present in similar amounts, or 14:0 was predominant (Albers et al. 1996). This difference may reflect an adaptation to the summer temperature in Lake Chasicó (25°C) and the warm Red Sea (22 to 25°C). The presence of 14:0 and 16:0 as the main fatty alcohols in wax esters of zooplankton species indicates generally omnivorous and opportunistic feeding behavior but also partly carnivorous feeding habits (Graeve et al. 1994a, Lee et al. 2006). On the basis of the lipid data and in agreement with the findings of Echaniz et al. (2006), who described *B. poopoensis* feeding on small algae but also on ciliates, rotifers and small nauplii, our

Table 1. Zooplankton abundance, organic carbon, lipid content and major lipid classes in Lake Chasicó. Seasonal mean and standard deviation from the 3 sampling locations. n: number of samples, WE: wax esters, TAG: triacylglycerols, PL: phospholipids

	Winter 2007 (n = 7)	Winter 2008 (n = 9)	Summer 2008 (n = 8)
Boeckella poopoensis (ind. l⁻¹)	37.7 ± 29.1	28.3 ± 9.5	54.2 ± 17.1
Moina eugeniae (ind. l⁻¹)	0.0 ± 0.0	0.0 ± 0.0	10.3 ± 9.3
Brachionus plicatilis (ind. l⁻¹)	0.0 ± 0.1	50.4 ± 34.6	44.2 ± 110
Organic carbon (μg C l⁻¹)	83.4 ± 25.5	120 ± 41.0	150 ± 73.3
Lipid mass (μg l⁻¹)	32.5 ± 6.2	42.3 ± 23.1	56.9 ± 26.5
Lipid content (% organic C)	40.7 ± 7.6	35.3 ± 9.0	38.2 ± 7.6
WE (% total lipid)[a]	59.2 ± 1.8	47.2 ± 5.5	44.6 ± 6.3
TAG (% total lipid)[a]	32.4 ± 3.9	37.3 ± 4.2	39.0 ± 5.5
PL (% total lipid)[a]	7.3 ± 4.0	10.5 ± 3.5	12.5 ± 4.9
[a]For lipid classes, n = 3			

Table 2. Composition of the major fatty acids and fatty alcohols (mass %) in total lipids of zooplankton and seston. Mean and standard deviation from the 3 sampling locations; n: number of samples; dashes: not detected

| | Zooplankton | | | Seston | | |
	Winter 2007 (n = 7)	Winter 2008 (n = 9)	Summer 2008 (n = 8)	Winter 2007 (n = 7)	Winter 2008 (n = 9)	Summer 2008 (n = 8)
Fatty acids						
14:0	5.0 ± 1.8	6.8 ± 0.7	6.6 ± 1.5	8.3 ± 4.2	12.2 ± 2.3	11.8 ± 3.7
15:0	2.6 ± 0.9	4.2 ± 0.6	4.1 ± 1.0	5.0 ± 2.8	5.6 ± 0.8	6.4 ± 1.5
16:0	13.0 ± 2.7	14.5 ± 1.2	18.2 ± 1.7	19.6 ± 2.4	18.5 ± 1.6	20.4 ± 2.3
16:1(n-7)	8.4 ± 2.9	11.1 ± 1.2	14.9 ± 3.5	14.1 ± 6.1	20.3 ± 3.8	21.6 ± 5.7
16:2(n-4)	1.4 ± 0.2	1.8 ± 0.7	1.6 ± 0.4	1.2 ± 1.1	3.1 ± 1.2	3.3 ± 2.1
16:3(n-4)	0.6 ± 0.1	0.6 ± 0.5	0.9 ± 0.5	0.6 ± 0.4	0.5 ± 0.4	0.8 ± 0.5
18:0	1.7 ± 0.5	2.1 ± 0.4	3.5 ± 0.5	11.5 ± 3.0	6.7 ± 1.5	6.0 ± 1.8
18:1(n-9)	1.7 ± 0.2	1.9 ± 0.5	3.8 ± 1.0	5.9 ± 3.1	6.0 ± 2.1	5.0 ± 1.5
18:1(n-7)	4.8 ± 0.5	5.7 ± 0.2	6.5 ± 1.1	5.9 ± 1.9	5.5 ± 0.5	8.2 ± 2.1
18:2(n-6)	1.6 ± 0.2	1.8 ± 0.2	3.0 ± 0.7	2.2 ± 1.0	2.5 ± 0.9	2.9 ± 0.7
18:3(n-3)	4.9 ± 0.8	5.5 ± 0.9	5.8 ± 2.7	2.0 ± 0.9	3.7 ± 1.3	3.0 ± 1.5
18:4(n-3)	17.9 ± 4.5	14.9 ± 2.0	3.3 ± 1.4	4.1 ± 1.6	3.7 ± 2.3	1.5 ± 0.6
20:4(n-6)	0.8 ± 0.2	0.9 ± 0.2	2.3 ± 0.4	0.2 ± 0.3	0.2 ± 0.3	0.8 ± 0.6
20:4(n-3)	12.0 ± 4.8	5.0 ± 3.0	1.3 ± 0.7	0.6 ± 0.7	0.3 ± 0.4	0.5 ± 0.5
20:5(n-3)	6.6 ± 1.1	7.4 ± 2.3	9.4 ± 1.5	3.6 ± 1.4	3.6 ± 1.3	3.2 ± 1.3
22:6(n-3)	11.8 ± 1.9	11.2 ± 1.3	11.5 ± 2.5	3.3 ± 1.6	4.0 ± 1.0	2.4 ± 1.3
Fatty alcohols						
14:0	3.2 ± 0.5	4.5 ± 0.6	7.0 ± 3.6	–	–	–
16:0	81.4 ± 2.2	79.1 ± 2.0	80.6 ± 4.7	–	–	–
18:1(n-9)	3.8 ± 0.6	4.0 ± 0.5	3.7 ± 1.9	–	–	–
18:1(n-7)	11.6 ± 1.4	11.5 ± 1.1	8.2 ± 3.6	–	–	–

study corroborates the classification of *B. poopoensis* as an opportunistic feeding species.

The fatty acid and alcohol compositions of the zooplankton and seston samples are presented in Table 2. The polyunsaturated fatty acids, 18:3(n-3), 18:4(n-3), 20:4(n-3), 20:5(n-3) and 22:6(n-3), were clearly higher in zooplankton than in seston samples. The saturated fatty acids 14:0, 15:0, 16:0 and 18:0, together with the monounsaturated fatty acids 16:1(n-7), 18:1(n-7) and 18:1(n-9), were generally dominant in seston. The higher values of the 16:1(n-7) and 20:5(n-3) fatty acids in summer suggest zooplankton grazing on diatoms; however, other diatom markers such as 16:2(n-4), 16:3(n-4) and 16:4(n-1) were only minor or trace compounds. The cyanobacterial marker 18:2(n-6) showed slightly higher values in zooplankton and seston during summer. Strongly elevated proportions of 18:4(n-3) were detected in zooplankton during winter, but this compound was less pronounced in seston samples. The accumulation of 18:4(n-3) in zooplankton is consistent with the occurrence of small flagellates as an important food source. The tendency of these markers coincided generally with the abundance of phytoplankton assemblages in Lake Chasicó (Kopprio et al. 2010).

The most striking feature of *Boeckella poopoensis* lipids was the extraordinary accumulation of the 20:4(n-3) fatty acid in winter, reaching an average value of 12% of total lipids in the winter of 2007 and with a maximum of 16% in August 2007. This fatty acid correlated significantly with 18:4(n-3) (r = 0.83, p < 0.001) but was detected only in trace amounts in seston. The close relationship between the occurrence of these 2 fatty acids suggests the pathway of a 1-step chain elongation of the 18:4(n-3) to the 20:4(n-3) fatty acid (Fig. 2).

Table 3. Composition of the major fatty acids and fatty alcohols (mass %) in the wax esters and triacylglycerols of zooplankton during winter (August 2007 and 2008). Data are mean ± SD from the 3 sampling locations; dashes: not detected

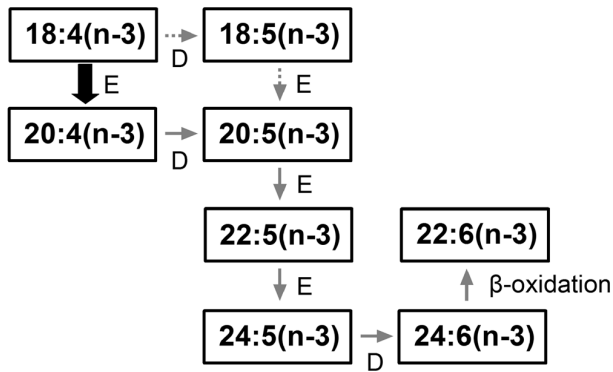|	Wax esters	Triacylglycerols
Fatty acids		
14:0	1.0 ± 0.9	8.0 ± 4.9
15:0	0.6 ± 0.6	4.7 ± 2.4
16:0	4.0 ± 1.2	19.8 ± 0.9
16:1(n-7)	13.6 ± 7.6	7.4 ± 3.2
16:2(n-4)	2.7 ± 2.4	1.2 ± 0.4
16:3(n-4)	0.4 ± 0.4	0.3 ± 0.3
18:0	3.7 ± 2.8	4.0 ± 2.1
18:1(n-9)	2.8 ± 1.0	2.4 ± 0.2
18:1(n-7)	6.5 ± 3.3	5.0 ± 0.7
18:2(n-6)	2.2 ± 2.1	1.5 ± 0.0
18:3(n-3)	7.5 ± 2.4	4.4 ± 1.1
18:4(n-3)	26.3 ± 9.4	20.2 ± 3.8
20:4(n-6)	0.5 ± 0.5	0.3 ± 0.2
20:4(n-3)	20.0 ± 4.3	7.4 ± 2.8
20:5(n-3)	1.6 ± 2.2	3.0 ± 1.4
22:6(n-3)	0.9 ± 1.0	4.4 ± 2.2
Fatty alcohols		
14:0	1.5 ± 1.3	–
16:0	85.4 ± 4.7	–
18:1(n-9)	2.4 ± 0.5	–
18:1(n-7)	9.0 ± 1.7	–

Fig. 2. Possible elongation pathway from the dietary 18:4(n-3) fatty acid to the major end product 20:4(n-3) and further elongation to 20:5(n-3) and 22:6(n-3). D: desaturation, E: elongation, bold arrow: major pathway, solid grey arrow: possible further E and D pathway, dashed grey arrow: unlikely pathway. Modified from Dalsgaard et al. (2003)

The very high amount of 18:4(n-3) in the storage lipids (mean of 26% in wax esters and 20% in triacylglycerols) during winter shows its high retention from the diet (Table 3). The 18:4(n-3) fatty acid is recommended as a trophic marker because it is usually incorporated unchanged into the lipids of zooplankton feeding on 18:4-rich flagellates (e.g. Reuss & Poulsen 2002, Dalsgaard et al. 2003). It is a nonessential fatty acid and can be rapidly replaced when the diet changes (Graeve et al. 1994b). The 20:4(n-3) occurred mainly in the long-term storage lipids (mean 20%, ranging from 15 to 24% in wax esters) but also in smaller amounts in triacylglycerols (mean 7%, ranging from 4 to 10%). There is usually no mention of the 20:4(n-3) fatty acid in zooplankton lipid compositions because it is either lacking altogether or only found in trace amounts. Von Elert (2004) suggests 20:4(n-3) as an indicator of polyunsaturated fatty acid biosynthesis in *Daphnia galeata*. However, only some aquatic species have the ability to biosynthesize polyunsaturated fatty acids (reviewed by Bell & Tocher 2009).

The elongation pathway used to produce the 20:4(n-3) fatty acid has been reported for the heterotrophic flagellate *Aulacomonas submarina*, which has the ability to extend 18:4(n-3) from its *Rhodomonas lacustris* diet into 20:4(n-3) (Bec et al. 2003b), giving rise to amounts of the latter as large as those found in *Boeckella poopoensis*. Another indicator of the possible chain elongation capability of *B. poopoensis* may be the elevated amount of the 18:1(n-7) fatty acid, which probably originated in the 16:1(n-7) fatty acid (Sargent & Henderson 1986, Hirche et al. 2003), which was highly abundant in the seston samples (Table 2). The second most important alcohol in

the zooplankton, 18:1(n-7), is mainly synthesized from the corresponding fatty acid by reduction and may also support the chain elongation concept. The same biosynthetic pathway has been proposed for the lipid-rich northern brackish water species *Limnocalanus macrurus* (Vanderploeg et al. 1998), a species related to *B. poopoensis*, with an even further elongation to the 20:1(n-7) fatty acid and alcohol (Hirche et al. 2003).

The grazing of *Boeckella poopoensis* on heterotrophic protozoa, which have the capacity to elongate the dietary fatty acid 18:4(n-3), might partially explain the occurrence of 18:4(n-3) and the corresponding 20:4(n-3) fatty acid. Non-flagellated Chlorophyceae may also contribute to the high amount of 20:4(n-3) (Léveillé et al. 1997), but *Oocystis* spp. presented elevated abundance only during the warmer months (Kopprio et al. 2010). The low percentage of 20:4(n-3) in seston (<1%) does not suggest a higher contribution to the zooplankton fatty acids. However, because copepods are capable of selective raptorial feeding (Kainz et al. 2009) *B. poopoensis* might choose organisms rich in polyunsaturated lipids as prey.

The chain elongation or accumulation of 20:4(n-3) produces fatty acids of higher calorific value (Albers et al. 1996), which, together with the accumulation of wax esters, provide a considerable energy store enabling survival through the more food-scarce winter period and fuelling gonad development and egg production throughout the year. The 20:4(n-3) fatty acid may have essential functions related to the growth and reproduction of zooplankton species. The n-3 polyunsaturated fatty acids are important for copepod reproduction (Jónasdóttir et al. 2009), and 20:4(n-3) has been reported to be strongly correlated with the growth of *Daphnia* spp., even though it only occurred in trace amounts (Becker et al. 2004). It may be speculated that the 20:4(n-3) fatty acid is also further elongated and desaturated to 20:5(n-3) and 22:6(n-3) (Fig. 2) to meet the growth and development needs of these copepods.

CONCLUSIONS

The present study has established that *Boeckella poopoensis* is able to accumulate extraordinarily high amounts of 18:4(n-3) and is likely able to elongate this fatty acid to 20:4(n-3). This is the first description of the accumulation of such high amounts of 20:4(n-3) in storage lipids of zooplankton species. It is likely that the unusual fatty acid composition, combined with the massive production of wax esters,

is an adaptation to the saline marine-like environment of Lake Chasicó: high wax ester content is known to be a feature especially of marine zooplankton species. It may also be speculated that *B. poopoensis* was originally an inhabitant predominantly of marine waters, where it is still occasionally found, and that it has become acclimatized to the saline conditions of Lake Chasicó after its introduction there. The tentative fatty acid biosynthesis of *B. poopoensis* is probably a strategy enabling it to thrive under extreme salt lake conditions, such as the highly variable food supply and strong temperature and salinity gradients.

The role and biological life-cycle implications of the unusually high concentration of 20:4(n-3) in the storage lipids of *Boeckella poopoensis* are still not clear. Experimental studies will help to resolve the ability of *B. poopoensis* to synthesize polyunsaturated fatty acids and to elucidate the essential functions of the 20:4(n-3) fatty acid.

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