

## Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids

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### Abstract

A new method that allows the highly reproducible supplementation of free fatty acids to planktonic microalgae was used to investigate the role of particular highly unsaturated fatty acids (PUFAs) in somatic growth limitation of *Daphnia galeata* feeding on *Scenedesmus obliquus* or *Stephanodiscus hantzschii*. No evidence for biotransformation of the supplemented fatty acids into other fatty acids by the algae was found. Using the algal cell itself as a transfer vehicle, the supplemented fatty acids were incorporated by *D. galeata*. In standardized growth experiments with juvenile *D. galeata*, growth on *S. obliquus* was improved by supplementation with the PUFAs  $\alpha$ -linolenic acid ( $\alpha$ -LA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3), but not by arachidonic acid (ARA, 20:4n-6), which illustrates that PUFAs should not be regarded as a single resource. Corresponding changes in the fatty acid pattern of *D. galeata* indicated that EPA is the limiting PUFA during growth on *S. obliquus* and that *D. galeata* converts DHA and C<sub>18</sub>-PUFAs into EPA. Growth on *S. hantzschii* was not improved by supplementation with EPA and ARA but was with  $\alpha$ -LA, which indicates that  $\alpha$ -LA is the limiting PUFA and that EPA cannot be converted into  $\alpha$ -LA. These results suggest that the availability of EPA determines which PUFA is limiting for growth. Because of the ability of the daphnids to convert  $\alpha$ -LA into EPA, both PUFAs are substitutable resources under EPA limitation, but because EPA cannot be converted into  $\alpha$ -LA, both PUFAs are nonsubstitutable resources under  $\alpha$ -LA limitation.

In aquatic food webs, the factors that regulate energy transfer between primary producers and consumers are crucial in understanding the transfer of energy across the plant–herbivore interface. It has been clear for many years that variation in the carbon transfer efficiency from primary to secondary production is quite large. This variation can be attributed to variation in food quality, but the determinants of food quality might be of a different nature, such as morphology, digestive resistance, toxicity, and nutritional inadequacy.

From a nutritional point of view, not all units of carbon are equal. Nutrient-limited algae (in freshwater systems mostly P-limited) are widely accepted to be a food source of low quality (Sterner and Schulz 1998). However, at C:P ratios <300, food quality for *Daphnia* might become constrained by factors other than P (Sundbom and Vrede 1997). Unless mineral limitation, toxins, or algal morphology constrain the utilization of algal biomass, the quality of algal carbon determines carbon transfer efficiency. Low quality of carbon can be due to a shortage of essential biochemicals in the diet, since such nutrients cannot be synthesized or are synthesized by a consumer in amounts inadequate to sustain growth. Polyunsaturated fatty acids (PUFAs, fatty acids with two or more double bonds) are essential for many vertebrates and invertebrates (Stanley-Samuelson et al. 1988), and the importance of PUFAs in freshwater zooplankton nutrition has recently been articulated (Gulati and DeMott 1997). Re-

sults of experiments with monoalgal food have suggested that high food quality correlates with high long-chain PUFA content (Ahlgren et al. 1990; Müller-Navarra 1995a). Generally, taxa such as cryptophytes and chrysophytes (including diatoms) are rich in long-chain PUFAs, whereas cyanobacteria are a poor PUFA source (Ahlgren et al. 1992).

Evidence for PUFA limitation of *Daphnia* in nature is indirect and comes from correlations of somatic growth rates of *Daphnia* raised on natural lake seston with chemically determined sestonic parameters. In a field survey, growth of *Daphnia* correlated best with the sestonic content of phospholipids (De Lange and Arts 1999), which include the highest concentrations of n-3PUFAs. In the mesotrophic Lake Schöhsee, eicosapentaenoic acid (EPA, 20:5n-3) concentrations show the highest correlation with growth of *Daphnia*; this has been interpreted as EPA limitation (Müller-Navarra 1995b). However, in Lake Constance, EPA limitation has been experimentally ruled out by supplementation of seston with an EPA-rich diatom, and strong correlative evidence has suggested that  $\alpha$ -linolenic acid ( $\alpha$ -LA, 18:3n-3) constrains somatic growth of *Daphnia* on the seston (Wacker and von Elert 2001).

Correlative evidence, although useful, does not provide proof of causal limitation. Correlative evidence from a study of a hypereutrophic pond has suggested that the low food quality of the seston in the summer was due to limited availability of EPA caused by the dominance of cyanobacteria during that season (Müller-Navarra et al. 2000). However, this could not be experimentally supported in experiments with *Synechococcus elongatus*, which indicated that the dietary inadequacy of cyanobacteria is due to an unidentified non-PUFA lipid (von Elert and Wolffrom 2001). This led my laboratory to test experimentally the correlative evidence for food quality constrained by EPA or  $\alpha$ -LA in two mesotrophic lakes, Lake Schöhsee and Lake Constance, in

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which cyanobacteria are of minor importance for the phytoplankton. The finding that  $\alpha$ -LA or EPA seem to be limiting in different lakes has been proposed to be due to differences in availability of EPA in Lake Schöhsee and Lake Constance (Wacker and von Elert 2001). This reasoning implies the hypotheses that (1) single PUFAs can become limiting for somatic growth of *Daphnia* and that (2) the availability of particular PUFAs determines which is the limiting PUFA. Neither of these hypotheses has been experimentally tested.

The most straightforward means of determining whether a single substance in the food is limiting is to use dietary supplements. Supplementation of microalgae has so far been restricted to the addition of various particles or droplets, mainly as microcapsules or emulsions; unfortunately, in most cases only mixtures of PUFAs have been tested (DeMott and Müller-Navarra 1997; Weers and Gulati 1997; Goulden et al. 1999). Although the results from these approaches point to PUFAs being limiting for *Daphnia*, the particular limiting fatty acid was not identified. Single fatty acids have been supplemented exogenously to cyanobacteria (Williams et al. 1990; Quoc et al. 1994), but such supplementation has not been applied to studies of food quality and partly led to lysis of algal cells when using high concentrations of fatty acids (this study).

A newly developed method is presented here that allows the fatty acid profile of microalgae to be manipulated in a highly reproducible way. Single fatty acids are added using the algal cell itself as the transfer vehicle so that all other features of the food (e.g., morphology and toxicology) remain unaffected. This method is used here to investigate whether PUFAs can become limiting for the growth of *D. galeata* on eukaryotic algae and whether the availability of EPA determines which particular compound constrains food quality.

## Methods

**Culture conditions**—Laboratory growth experiments were carried out with a clone of *Daphnia galeata* SARS, originally isolated from Lake Constance (Stich and Lampert 1984). Mothers were grown in natural lake water (filtered through a 0.45- $\mu$ m pore-sized membrane) in temperature-controlled (20°C) flow-through chambers (250 ml), which were continuously supplied with a suspension of *Scenedesmus obliquus* MEYEN (strain SAG 276-3a, Stammsammlung für Algen, SAG, Göttingen, Germany) at a nonlimiting concentration (2 mg C L<sup>-1</sup>; flow rate, 60 ml h<sup>-1</sup>). *S. obliquus* was grown in a chemostat at a dilution rate of 0.5 d<sup>-1</sup> at 20°C (light, 120  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) in modified WC medium (Guillard 1975). *Stephanodiscus hantzschii* GRUNOW, which had been isolated from Lake Constance, was grown in a chemostat at 16°C (dilution rate, 0.25 d<sup>-1</sup>; light, 80  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) in WC medium with vitamins. Chemostat-grown cells of *S. obliquus* and *S. hantzschii* were concentrated by centrifugation and resuspended in WC medium. Carbon concentrations of these stock solutions of algae were estimated from photometric light extinction (800 nm) using carbon extinction equations. Particulate organic carbon (POC) was de-

termined with an NCS-2500 analyzer (Carlo Erba Instruments).

**Growth experiments**—The growth of *D. galeata* was monitored in two experiments: one with *S. obliquus* and one with *S. hantzschii* as algal food source. In a third experiment, *D. galeata* was raised on *S. obliquus* that had been supplemented with one of several fatty acids, and the fatty acids in *D. galeata* were analyzed. The daphnids used in these three experiments originated from one clutch of third-brood juveniles from mothers that had been raised under identical conditions for three generations. All treatments of an experiment were conducted with juveniles from the same cohort. Experimental juveniles were collected within 8 h of birth and grown for another 40 h in a flow-through system on 2 mg C *S. obliquus* cells L<sup>-1</sup>. The animals were then used in the growth experiments, which lasted from day 2 to day 6. Each test was carried out in 0.5 L of filtered lake water with 2 mg C algal food L<sup>-1</sup>; the food suspensions were renewed daily. Dry weights of the animals from day 2 and day 6 were used for the calculation of the somatic growth rate. Each test contained 10 daphnids and was carried out in triplicate. Somatic growth rates ( $g$ , d<sup>-1</sup>) were calculated from the equation

$$g = [\ln(W_t) - \ln(W_0)]/t$$

where  $W_0$  is the mean individual dry weight of a subsample of the animals at the beginning of the growth experiment and  $W_t$  is the mean individual dry weight of the daphnids after a duration of  $t = 4$  d. Dry weights were mean values of 10 individuals, weighed on an electronic balance (Mettler UMT 2) and recorded to the nearest 0.1  $\mu$ g. Growth rates were calculated as the means of each treatment. Growth on alga that had been incubated with bovine serum albumin (BSA) plus single fatty acids was compared with growth on the same alga that had been incubated with BSA.

**Fatty acid analysis**—For fatty acid analysis, aliquots of algal suspensions corresponding to approximately 0.5 mg POC were filtered on a precombusted GF/F filter (Whatman, 25-mm diameter). Lipids were extracted with dichloromethane/methanol (2:1, v/v) from loaded filters or from 50 *D. galeata* individuals. The lipid extract was evaporated to dryness with nitrogen and transesterified with 3 M methanolic HCl (Supelco) according to Mason and Waller (1964); the internal standards were heptadecanoic acid methylester and tricosanoic acid methylester. Details are given elsewhere (von Elert and Stampfl 2000). The final sample volume was 10  $\mu$ l.

Fatty acid methyl esters (FAMES) were analyzed by gas chromatography on an HP 6890 GC with the following configuration: column, DB-225 (J&W Scientific, 30 m  $\times$  0.25 mm ID  $\times$  0.25  $\mu$ m film); oven, 60°C (1 min) to 150°C at 30°C min<sup>-1</sup> then to 170°C at 3°C min<sup>-1</sup> then to 220°C at 2°C min<sup>-1</sup> and hold for 6 min; carrier, helium (35 cm s<sup>-1</sup>); detector, FID 250°C; injector, 250°C (total run time 42 min sample<sup>-1</sup>). A 1- $\mu$ l aliquot of each sample was injected splitlessly. FAMES were identified by comparison of retention times with those of reference compounds, by cochromatography with reference compounds, and by GC-MS. FAMES

were quantified by comparison to internal standards of known concentration and to response factors determined for each FAME from mixtures of known composition. It was not possible to distinguish between petroselinic acid (C18:1n-12) and oleic acid (C18:1n-9). The detection limit was 20 ng of fatty acid. The absolute amounts of each FAME were related to the independently determined POC content of the sample. Reference FAMES were purchased from Sigma and Supelco.

For the detection of EPA in *S. obliquus*, the evaporated lipid extract of the alga was saponified with 4 ml of 0.2 M methanolic KOH (1 h, 70°C), and nonsaponifiable lipids were removed by extracting three times with 2 ml of iso-hexane/diethylether (9:1, v/v). After acidification to pH 1 with HCl, free fatty acids were extracted three times with 2 ml of iso-hexane. This fraction was evaporated to dryness in a stream of nitrogen, and the residue was resuspended in 40  $\mu$ l CH<sub>2</sub>Cl<sub>2</sub> and 10  $\mu$ l of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) according to Jüttner (2001). After incubation for 1 h at ambient temperature, 1  $\mu$ l was injected into a gas chromatograph–mass spectrometer (Finnigan MAT GCQ) equipped with a fused-silica capillary column (DB-225MS, 30 m  $\times$  0.25 mm ID  $\times$  0.25  $\mu$ m film) and chromatographed as described above. For the detection of EPA in the EI ionization mode by single ion monitoring (SIM), the ions *m/z* [M-57], [M-39], and [M-149] were recorded. The detection limit in a real matrix was 1 ng of EPA.

**Supplementation with fatty acids**—Microalgae were enriched with a single fatty acid as follows. BSA (20 mg, Sigma) was dissolved in 5 ml of ultrapure water, and 400  $\mu$ l of an ethanolic stock solution of free fatty acid (2.5 mg ml<sup>-1</sup>) was added with gentle stirring. Then, 10 ml of WC medium and subsequently 4 mg POC of the algal stock solution were added, and the volume was brought to 40 ml with WC medium. The resulting suspension was incubated on a rotary shaker (100 rpm) for 4 h in the light (100  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). Algal cells from aliquots of the suspension corresponding to 1 mg POC were then concentrated by centrifugation, and the supernatant was discarded. In order to remove excess BSA and free fatty acid, the cells were washed twice in 10 ml of WC medium; the resulting 10 ml of algal suspension was used as food in the *D. galeata* growth experiments. Controls were incubated without adding BSA and fatty acids. The purity of BSA (Sigma A7906) was >98%; free fatty acids (Sigma) were  $\geq$ 99% pure. When the effect of externally added fatty acids on the fatty acid profile of *S. obliquus* or *S. hantzschii* was investigated, all treatments were carried out in triplicate in a single experiment for each alga. The integrity of algal cells was checked qualitatively using a standard microscope.

**Data analysis**—To determine the effect of incubating algae with single fatty acids on the fatty acid profile of the algae, each detected fatty acid was analyzed using analyses of variance; the content of each fatty acid was the dependent variable, and the kind of incubation was the independent variable. Analysis of variance was used to compare the effects of different diets on the PUFA content in *D. galeata*; PUFAs, exogenously supplemented or involved in fatty acid

anabolism, were the dependent variable, and diet was the independent variable. The dependent variable was log<sub>10</sub> transformed in order to meet the assumption of homogeneity of variance. A significance level of  $\alpha = 0.05$  was applied to the entire statistical analysis, and the number of analyses of variance performed was taken into account by sequential Bonferroni adjustment (Rice 1989). The effects of single treatments by post hoc tests (Tukey's HSD multiple comparison test) were tested on the same probability level as the respective analysis of variance. In cases where a particular fatty acid was not detectable, the samples were assumed to contain fatty acids at the detection limit and the coefficient of variance was assumed to be identical to that of samples in which the particular fatty acid had been detected.

## Results

**Supplementation of PUFA**—The effect of different incubation time in the presence of fatty acids was tested in *S. hantzschii*, which is rich in PUFAs. Incubating *S. hantzschii* in the presence of  $\alpha$ -LA—a constituent of the diatom—led to a higher content of this PUFA in *S. hantzschii*. Incubation with 30  $\mu$ M  $\alpha$ -LA over time showed that the resulting concentration of  $\alpha$ -LA in the biomass is a function of the incubation time; saturation was reached after 10 to 15 h (Fig. 1A). No difference was found between experiments in which *S. hantzschii* was incubated in the light or in the dark, which indicated that the enrichment of  $\alpha$ -LA is not a photosynthesis-mediated process. Furthermore, the resultant concentration of  $\alpha$ -LA in the algal biomass was a linear function of the concentration of  $\alpha$ -LA in the incubation medium (Fig. 1B). Hence, varying the concentration of the externally added free fatty acid or varying the time of incubation provides two independent means to affect the resultant fatty acid concentration in the target cell. Results from replicate incubations of *S. hantzschii* with a given external concentration of  $\alpha$ -LA showed that the coefficient of variance (CV = SD  $\times$  100/mean) found for concentrations of  $\alpha$ -LA in the diatom was always <10% and hence within the same range as for other nonsupplemented fatty acids. This indicated that the enrichment technique provides a highly reproducible means of manipulating the fatty acid profile of algal cells.

This technique was applied to investigate how specifically the fatty acid profile of *S. obliquus*, a widely used algal food for the maintenance of *Daphnia*, is affected by the exogenous supplementation of a single fatty acid. *S. obliquus* is relatively rich in C<sub>18</sub>-PUFAs, with  $\alpha$ -LA being the most abundant PUFA (Table 1). No long-chain PUFAs (>C<sub>18</sub>) were detectable. Although the retention time of peaks suggested that EPA and docosahexaenoic acid (DHA, 22:6n-3) were present, this was ruled out by mass spectrometry and by cochromatography with reference compounds. Fatty acid 20:4n-3 was also not detectable (GC-MS, SIM mode). Incubation with BSA did not affect the content of any fatty acid in *S. obliquus* (Table 1). Incubation with  $\alpha$ -LA significantly increased the natural content of this PUFA in *S. obliquus* (Tukey's HSD,  $p < 0.001$  following ANOVA,  $F_{5,12} = 46.53$ ;  $p < 0.001$ ) without affecting any other fatty acid in the alga (Table 1). When *S. obliquus*, which lacks the long-

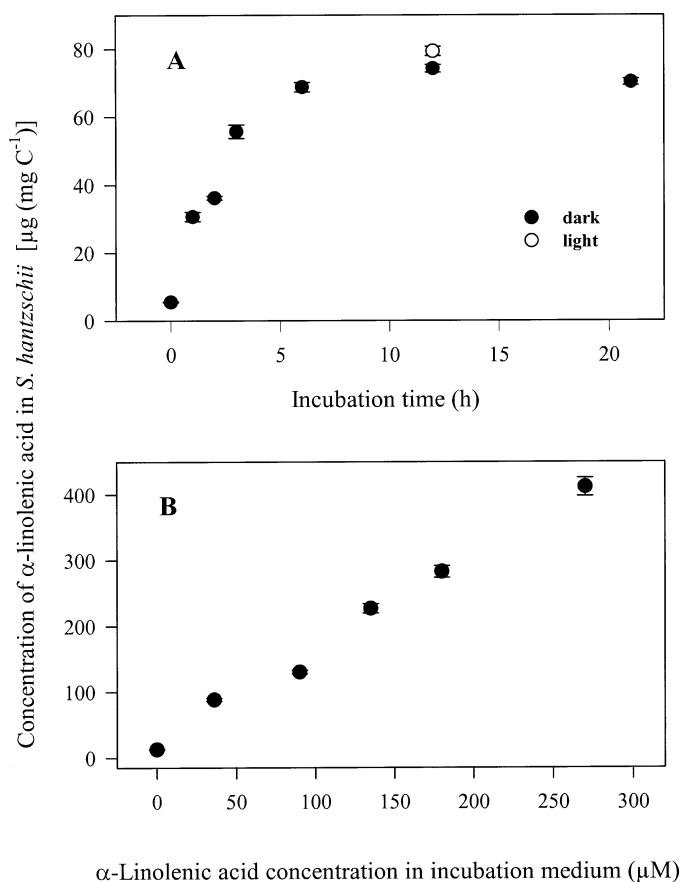


Fig. 1. Concentration of  $\alpha$ -linolenic acid ( $\alpha$ -LA, 18:3n-3) in *Stephanodiscus hantzschii* after incubation in the presence of  $\alpha$ -LA. (A) Effect of the incubation time with 30  $\mu\text{M}$   $\alpha$ -LA. (B) Effect of the concentration of  $\alpha$ -LA in the incubation medium with an incubation time of 4 h. Values are means  $\pm$  SE ( $n = 3$ ). Results of incubation in the dark were similar to those in the light.

chain PUFA EPA, was incubated with EPA under standard conditions, the EPA concentration increased to the level of the naturally most abundant PUFA  $\alpha$ -LA (Table 1, Tukey's HSD,  $p < 0.001$  following ANOVA,  $F_{5,12} = 2,517$ ;  $p < 0.001$ ). Total fatty acids increased by a factor of 1.3. No significant changes in the content of other fatty acids were observed, which indicates that EPA was not transformed into other fatty acid species by the alga (Table 1). Incubation of *S. obliquus* with the long-chain PUFA DHA, which is not detectable in this alga, significantly increased the DHA content to 30  $\mu\text{g (mg C}^{-1}\text{)}$  (Tukey's HSD,  $p < 0.001$  following ANOVA,  $F_{5,12} = 6,418$ ;  $p < 0.001$ ), with DHA representing 10% of total fatty acids; again, no indications for a biotransformation of DHA into a different fatty acid by *S. obliquus* were found (Table 1). Similarly, incubation with a monoenic fatty acid (18:1n-7) (Tukey's HSD,  $p < 0.001$  following ANOVA,  $F_{5,12} = 85.85$ ;  $p < 0.001$ ) and with the n-6 long-chain PUFA arachidonic acid (ARA, 20:4n-6) (Tukey's HSD,  $p < 0.001$  following ANOVA,  $F_{5,12} = 5,912$ ;  $p < 0.001$ ) resulted in significant increases of the concentrations of these compounds, whereas the levels of other fatty acids were not affected (Table 1). Microscopy revealed no indi-

cation for cell lysis, which indicates that the added PUFAs were not toxic to the algal cells.

**Feeding experiments with *Scenedesmus***—When 2-d-old neonates of *D. galeata* were grown for 4 d on *S. obliquus*, which had been supplemented with one of several fatty acids, the somatic growth of the animals showed significant differences (ANOVA,  $F_{5,12} = 9.89$ ;  $p < 0.001$ ). Compared to the growth rate on *S. obliquus* not incubated with a PUFA ( $0.47 \text{ d}^{-1}$ ), the growth rates of the daphnids increased significantly when fed on *S. obliquus* incubated with one of the three n-3 PUFAs:  $\alpha$ -LA (Tukey's HSD,  $p = 0.024$ ), EPA (Tukey's HSD,  $p = 0.005$ ), and DHA (Tukey's HSD,  $p = 0.0006$ ) (Fig. 2). Incubation of *S. obliquus* with the monoenic fatty acid 18:1n-9 (Tukey's HSD,  $p = 0.21$ ) or with the n-6 PUFA ARA (Tukey's HSD,  $p = 0.59$ ) had no significant effect on the growth of *D. galeata*; hence, the growth-enhancing effect was not caused by an unspecific fatty acid, but restricted to n-3 PUFAs. The PUFAs added to *S. obliquus* had no toxic effects on *D. galeata*: in no case did supplementation with PUFAs cause a depression of growth of the daphnids.

In order to investigate whether the growth-enhancing effects of  $\alpha$ -LA, EPA, and DHA were due to a colimitation of *Daphnia* or resulted from a conversion of these n-3 PUFAs to a common metabolite that was limiting the growth of *D. galeata*, the effects of higher contents of  $\text{C}_{18}$ -PUFAs and long-chain PUFAs in *S. obliquus* on the content of these compounds in *D. galeata* were investigated. Feeding daphnids with *S. obliquus* enriched with EPA, which was not detectable in unsupplemented *S. obliquus*, significantly increased the EPA content of the animals 140-fold compared to control animals (Fig. 3D), which showed that the EPA supplemented to *S. obliquus* was assimilated by the animals. Despite the substantial increase of EPA in *D. galeata* feeding on EPA-enriched alga, no increases in other  $\text{C}_{18}$ -PUFAs and long-chain PUFAs were detected (Fig. 3). DHA was not detectable in these daphnids; therefore, the EPA assimilated did not serve as a precursor for the synthesis of DHA in the animals. The levels of putative EPA precursors (20:4n-3 and 18:4n-3) in these daphnids were significantly lowered (Fig. 3B,C).

Feeding the daphnids with *S. obliquus* enriched with DHA, which was not detectable in unsupplemented *S. obliquus*, led not only to the presence of DHA in the animals but also to an EPA level that exceeded that of DHA almost by a factor of five (Fig. 3D,E). This shows an internal conversion of DHA to EPA by *D. galeata*. At the same time, significantly lower levels of  $\text{C}_{18}$ -PUFAs (18:3n-3 and 18:4n-3) and of 20:4n-3 (Fig. 3A–C) were found in the animals, which showed that DHA was not converted into  $\text{C}_{18}$ -PUFAs or 20:4n-3 and that DHA reduced the assimilation of putative precursors (18:3n-3 and 18:4n-3) and the synthesis of intermediates (20:4n-3) of EPA synthesis.

*D. galeata* grown on unsupplemented *S. obliquus* not only contained the  $\text{C}_{18}$ -PUFAs  $\alpha$ -LA and stearidonic acid (SA, 18:4n-3), which are abundant in the green alga, but also the long-chain PUFAs 20:4n-3 and EPA (Fig. 3C,D), which were not detectable in *S. obliquus* (Table 1). To determine whether these long-chain PUFAs in *D. galeata* originate

Table 1. Fatty acid content of *Scenedesmus obliquus* before and after incubation with BSA plus one of several PUFAs or with BSA only. Controls were incubated without BSA and fatty acids. Fatty acid contents that changed significantly after incubation of *S. obliquus* are given in bold. Values are means of three independent incubations ( $n=3$ ). Coefficient of variance (CV) =  $SD \times 100/\text{mean}$ . nd, not detectable.

Fatty acid	Fatty acid content ( $\mu\text{g (mg C)}^{-1}$ ) (CV)						
	Control	Incubation with BSA			Incubation with BSA plus PUFA		
		18:1n-7	18:3n-3	20:4n-6	20:5n-3	22:6n-3	
14:0	2.3(3.6)	2.5(5.7)	2.3(3.1)	2.4(1.5)	2.1(0.8)	2.2(5.4)	2.1(9.4)
16:0	53.5(5.8)	56.2(5.2)	57.6(4.4)	61.1(1.4)	55.0(6.3)	54.8(8.5)	53.7(3.7)
16:1n-7	0.9(3.5)	0.9(3.2)	1.1(9.1)	0.9(0.3)	0.8(2.6)	0.9(3.7)	0.8(2.6)
18:0	10.0(5.4)	10.0(3.5)	10.5(3.5)	10.3(1.9)	10.7(9.1)	10.5(7.3)	10.4(3.2)
18:1n-12/1n-9	76.1(7.5)	78.7(6.6)	83.6(4.5)	82.1(1.7)	78.5(9.5)	79.5(9.4)	79.4(4.3)
18:1n-7	1.3(6.1)	1.4(5.0)	<b>2.9(3.9)</b>	1.4(3.3)	1.3(6.7)	1.3(6.8)	1.1(9.5)
18:2n-6	26.1(8.1)	27.9(6.6)	29.2(6.2)	28.8(9.6)	23.1(10.4)	25.1(9.8)	27.5(5.0)
18:3n-6	1.4(5.5)	1.5(3.2)	1.5(3.7)	1.5(9.0)	1.2(9.4)	1.4(4.8)	1.5(7.7)
18:3n-3	73.6(8.7)	76.9(7.1)	80.3(8.5)	<b>162.8(3.0)</b>	69.9(11.3)	69.4(11.1)	72.5(1.1)
18:4n-3	6.2(8.2)	6.6(4.7)	6.6(4.9)	7.4(8.5)	6.8(10.7)	5.5(7.6)	5.8(2.8)
20:1n-9	1.0(4.4)	1.0(4.3)	1.1(3.2)	1.2(8.6)	1.1(5.6)	1.2(5.2)	1.1(8.0)
20:4n-6	nd	nd	nd	nd	<b>31.8(11.5)</b>	nd	nd
20:5n-3	nd	nd	nd	nd	nd	<b>73.7(9.5)</b>	nd
22:0	1.9(4.8)	2.0(4.5)	2.0(3.8)	2.2(8.1)	2.6(8.9)	2.4(7.3)	2.0(7.8)
22:6n-3	nd	nd	nd	nd	nd	nd	<b>29.5(4.9)</b>

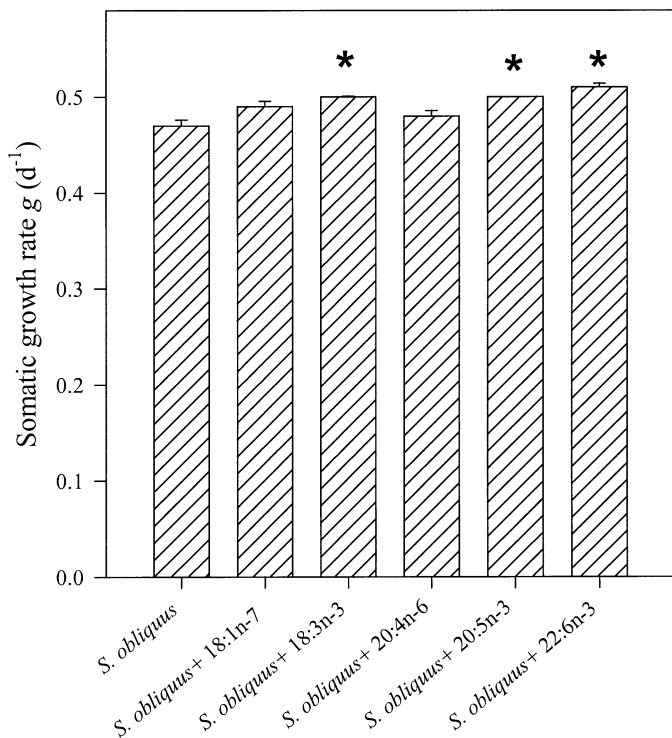


Fig. 2. Effects of incubation of *Scenedesmus obliquus* with single fatty acids on the somatic growth rate of *Daphnia galeata* fed on the alga. Food concentration was  $2 \text{ mg POC L}^{-1}$ . Data are means  $\pm$  SE for three replicates per treatment. Asterisks indicate treatments significantly different (Tukey's HSD test,  $p < 0.05$ ) from feeding on unsupplemented *S. obliquus* (control).

from  $\text{C}_{18}$ -PUFAs assimilated from *S. obliquus* and subsequently are elongated and desaturated by the grazer, the alga was supplemented with SA, and the amount of EPA and of intermediates in the synthesis of EPA from  $\text{C}_{18}$ -PUFAs in the animals were measured. Supplementation of *S. obliquus* with SA led to a fourfold increase in the amount of SA in *S. obliquus* (data not shown). In animals raised on this food, the content of SA, 20:4n-3, and EPA increased significantly. The SA content in the animals increased fourfold (Fig. 3B), the 20:4n-3 content increased threefold (Fig. 3C), and the EPA content increased twofold (Fig. 3D), whereas the DHA content did not increase; this indicated that  $\text{C}_{18}$ -PUFAs are converted to EPA and thus explains the growth-enhancing effect of  $\alpha$ -LA-enriched *S. obliquus* on *D. galeata*.

**Feeding experiments with *Stephanodiscus***—To test whether the ameliorating effect of  $\text{C}_{18}$ -PUFAs and EPA on the food quality of *S. obliquus* can be generalized for other food sources containing a high amount of EPA, the effect of supplementing the diatom *S. hantzschii* with  $\alpha$ -LA and EPA on the growth of *D. galeata* was investigated. In contrast to *S. obliquus*, the diatom contains substantial amounts of EPA (24% of total fatty acids, Table 2) and only low amounts of  $\alpha$ -LA (2.4%) and SA (2.1%). Incubation of *S. hantzschii* with  $\alpha$ -LA or EPA significantly increased the content of the respective supplementary fatty acid ( $\alpha$ -LA: Tukey's HSD,  $p < 0.0001$  following ANOVA,  $F_{2,6} = 3,946.05$ ;  $p < 0.0001$ ; EPA: Tukey's HSD,  $p < 0.0001$  following ANOVA,  $F_{2,6} = 186.3$ ;  $p < 0.0001$ ). No changes in the content of the other fatty acids were observed (Table 2), which showed that the diatom did not transform  $\alpha$ -LA or EPA. Hence, supplementation with  $\alpha$ -LA or EPA led to an increased content of only the added PUFAs. Somatic growth of *D. galeata* was clearly affected by the kind of supplementation given to *S. hantz-*

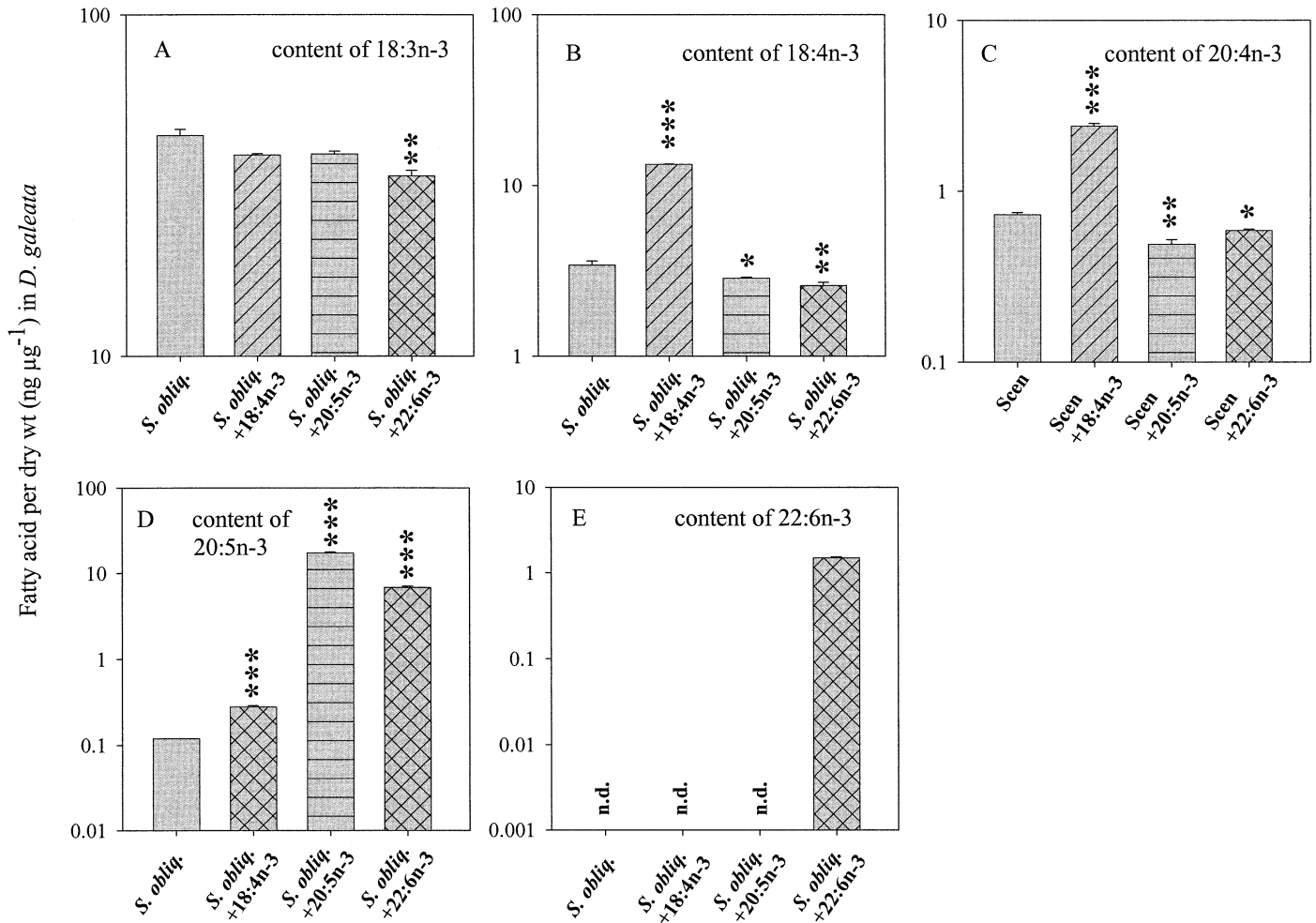


Fig. 3. Content of (A) 18:3n-3, (B) 18:4n-3, (C) 20:4n-3, (D) 20:5n-3, and (E) 22:6n-3 in *Daphnia galeata* as a function of supplementation of *Scenedesmus obliquus* with different single PUFAs (depicted on x-axes). *S. obliquus* was supplemented with stearidonic acid (18:4n-3), eicosapentaenoic acid (20:5n-3), or docosahexaenoic acid (22:6n-3). Data are means + SE for three replicates per treatment. Fatty acid contents significantly different from that of controls (Tukey's HSD test) are depicted as \*  $p < 0.05$ , \*\*  $p < 0.005$ , and \*\*\*  $p < 0.0005$ .

*schii* (Fig. 4; ANOVA,  $F_{2,6} = 12.88$ ,  $p < 0.01$ ). The increased content of EPA in *S. hantzschii* did not affect growth (Tukey's HSD,  $p = 0.85$ ), but growth was constrained by the availability of  $\alpha$ -LA (Fig. 4; Tukey's HSD,  $p = 0.0085$ ).

## Discussion

The most direct way to disentangle the roles of covarying food parameters independent of food quantity is to supplement the diet with that particular component and to look for changes in an appropriate life history parameter (e.g., growth). A variety of methods are available: from marine aquaculture (Coutteau and Sorgeloos 1997). Microcapsules made from alginate and gelatin (Sundbom and Vrede 1997) or protein (Goulden et al. 1999), suspensions of very small oil droplets (DeMott and Müller-Navarra 1997) or emulsions of defined mixtures with unknown emulsifiers (Weers and Gulati 1997) have been used. These methods might lead to erroneous results either by improving the performance of the

diet through increasing the water stability of food particles or by interfering with the retention of the dietary supplements (Coutteau et al. 1997).

For the nonselective grazer *D. galeata*, the supplemented fatty acids were provided as part of the food particle without the use of emulsifiers to make detailed studies on the role of particular fatty acids in food quality and in biosynthetic conversions within the grazer feasible. Fatty acids have been supplemented exogenously with cyanobacteria (Williams et al. 1990; Quoc et al. 1994). Using this approach, insolubility of fatty acids occurred, which was noted to lead to lysis of algal cells (this study, not quantified), probably because of the toxicity of free fatty acids (Ikawa 1989). Therefore, fatty acids were added as BSA complexes by modifying the method of Spector and Hoak (1969): instead of adding a particulate adsorbent coated with the free fatty acid to the BSA solution, ethanolic solutions of the free fatty acids were added directly to the BSA solution. Adding fatty acid-BSA complexes to algal cells enhanced the solubility of the fatty

Table 2. Fatty acid content of *Stephanodiscus hantzschii* after incubation with BSA only or with BSA plus PUFA. Fatty acid contents that changed significantly after incubation of *S. hantzschii* with single PUFAs are given in bold. Values are means of three independent incubations ( $n=3$ ). Coefficient of variance (CV) =  $SD \times 100/\text{mean}$ . nd, not detectable.

Fatty acid	Fatty acid content ( $\mu\text{g (mg C)}^{-1}$ ) (CV)		
	Incubation with BSA	Incubation with BSA plus 18:3n-3	Incubation with BSA plus 20:5n-3
16:0	90.8(2.8)	88.7(4.2)	87.1(4.1)
16:1n-7	223.8(4.5)	206.8(10.8)	198.5(9.3)
17:1n-7	10.5(4.1)	10.2(10.5)	9.9(3.4)
18:0	5.8(4.9)	6.8(10.8)	6.1(2.0)
18:1n-12/1n-9	4.4(5.4)	4.3(6.6)	4.2(0.8)
18:1n-7	2.0(3.9)	1.8(10.3)	1.9(8.2)
18:2n-6	10.3(4.3)	10.6(7.5)	10.2(4.2)
18:3n-6	1.7(5.8)	1.6(12.4)	1.5(7.8)
18:3n-3	12.7(4.0)	<b>226.7(5.4)</b>	12.6(4.2)
18:4n-3	10.0(5.2)	10.0(6.4)	9.0(3.9)
20:0	1.1(2.2)	1.0(3.4)	1.0(1.4)
20:4n-6	0.8(1.5)	0.8(6.7)	0.8(7.4)
20:5n-3	124.1(5.1)	116.5(6.5)	<b>269.0(6.5)</b>
22:0	1.1(2.2)	1.1(2.0)	1.0(3.9)
22:1n-9	1.3(4.8)	1.3(1.8)	1.3(7.3)
22:2n-6	1.5(3.9)	2.1(7.6)	1.9(9.1)
22:6n-3	21.3(7.7)	23.3(12.7)	19.8(8.1)
24:0	1.1(4)	1.2(1.1)	1.2(11.1)

acids, thereby avoiding the formation of micelles in the aqueous environment and toxic effects on algal cells. Williams et al. (1990) have shown initial rapid incorporation of exogenously added free fatty acids into the neutral lipid fraction after 24 h; however, in the current study, it was not investigated whether the supplemented fatty acids were associated with the surface of the algal cell or were incorporated into the algal lipids.

To date, attempts to investigate whether PUFAs are limiting growth of *D. galeata* on monoalgal food have mainly employed mixtures rather than single fatty acids. Growth-promoting effects on *Daphnia* were observed after addition of a PUFA-rich emulsion or fish oil to *Scenedesmus* (Sundbom and Vrede 1997; Weers and Gulati 1997) and after supplementation of *S. elongatus* with fish oil (DeMott and Müller-Navarra 1997). The latter was attributed to the high content of long-chain PUFAs in fish oil (however, see von Elert and Wolffrom 2001). In the only study in which the role of single fatty acids has been addressed, no effect on the somatic growth of *D. galeata* was observed (Sundbom and Vrede 1997), and it remains unclear whether the micro-encapsulated fatty acids were nutritionally available to the grazers because, unfortunately, fatty acids in the animals were not determined.

However, despite the evidence for PUFAs limiting growth of *D. galeata*, these fatty acids obviously should not be regarded as a single resource because the long-chain PUFA ARA had no effect on the growth of *D. galeata* on *S. obliquus*. The food-quality-enhancing effects of the three PUFAs  $\alpha$ -LA, EPA, and DHA on *S. obliquus* corroborate the

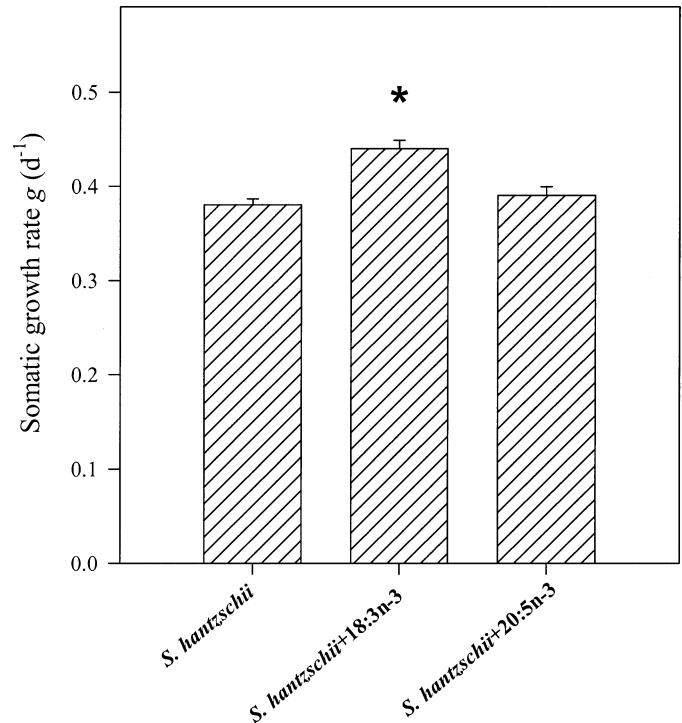


Fig. 4. Effects of incubation of *Stephanodiscus hantzschii* with single fatty acids on the somatic growth rate of *Daphnia galeata*. Food concentration was  $2 \text{ mg POC L}^{-1}$ . Data are means + SE for three replicates per treatment. Asterisks indicate treatments that are significantly different (Tukey's HSD test,  $p < 0.05$ ) from feeding on unsupplemented *S. hantzschii* (control).

above-mentioned ameliorating effects of PUFA mixtures. Additional EPA in *S. obliquus* only increased the EPA content in *D. galeata*, which indicates that EPA is not converted to another fatty acid and thus suggests that EPA is the growth-limiting fatty acid. This is supported by the retro-conversion of DHA to EPA in *D. galeata*, which is in agreement with Weers et al. (1997). This retroconversion is well known in *Artemia franciscana* and *Brachionus plicatilis*, which have been shown to catabolize DHA selectively during starvation (Navarro et al. 1999).

Similarly, the growth-promoting effect of  $\alpha$ -LA is probably due to a conversion of  $\alpha$ -LA to EPA. Although widespread in animals, it is not known whether these chain elongation and desaturation steps take place in freshwater cladocerans. The presence of 20:4n-3 and EPA in *D. galeata* raised on food lacking detectable amounts of these long-chain PUFAs and the increase in the EPA content after feeding on *S. obliquus* supplemented with SA suggest that daphnids are able to convert (n-3) $\text{C}_{18}$ -PUFAs to EPA.

The results of this study indicate that, in *D. galeata*, (n-3) $\text{C}_{18}$ -PUFAs can be converted to EPA, which is the limiting PUFA during growth on *S. obliquus*. Because EPA is limiting the growth of *D. galeata*, even though the potential EPA precursor  $\alpha$ -LA is the major PUFA in *S. obliquus*, the rate of conversion must be too low to meet demands. This is supported by an increase in growth and in concentrations of intermediates of EPA synthesis when  $\text{C}_{18}$ -PUFAs are administered and is in agreement with observed low conver-

sion capacities of unsaturated fatty acids in other aquatic invertebrates (e.g., Langdon and Waldock 1981).

Differences in the kind of limiting PUFA when feeding on *S. obliquus* and *S. hantzschii* indicate that the availability of EPA in the diet determines which particular PUFA is limiting. In *D. galeata* raised on a low-EPA alga such as *S. obliquus*, EPA will be limiting, whereas in animals raised on a high-EPA alga such as *S. hantzschii*, a different n-3 PUFA is limiting—in this case,  $\alpha$ -LA. This suggests (1) that EPA cannot be converted to  $\alpha$ -LA, which is in agreement with the content of  $\alpha$ -LA in daphnids fed on *S. obliquus* supplemented with EPA and (2) that the physiological role of  $\alpha$ -LA, albeit unknown, is distinct from the functions of EPA. A similar finding has been reported for the waxmoth *Galleria melonella*, which requires dietary EPA and  $\alpha$ -LA for normal development of adults (Dadd 1983) and which has the capability of converting  $\alpha$ -LA to EPA (Stanley-Samuelson 1987); on a diet rich in EPA, but free of  $\alpha$ -LA, morphologically abnormal adults develop. This nutritional deficiency in  $\alpha$ -LA can be compensated by C20:3n-3 and C22:3n-3, which are converted to  $\alpha$ -LA, whereas EPA cannot serve as a precursor for  $\alpha$ -LA because of the high degree of unsaturation (Stanley-Samuelson and Dadd 1984).

At first glance, correlative field data would have suggested much stronger effects of EPA supplementation than found in this study. For the putatively EPA-limited seston in Lake Schöhsee, Müller-Navarra (1995b) calculated an EPA saturation concentration for the growth of *D. galeata* of  $0.8 \mu\text{g C L}^{-1}$  and observed an absolute difference of  $0.28 \text{ d}^{-1}$  in the growth rate when the EPA content of the seston differed by approximately  $1 \mu\text{g C L}^{-1}$ . In the current study, the growth rate of *D. galeata* on *S. obliquus* increased significantly by  $0.03 \text{ d}^{-1}$ , with a difference in the EPA content of the food by  $>150 \mu\text{g C L}^{-1}$ . However, the effect of EPA supplementation was small because the daphnids already had a somatic growth rate close to the maximum growth rate possible on unsupplemented *S. obliquus* ( $0.47 \text{ d}^{-1}$ ). Growth as a function of food availability is a saturation function with a decreasing slope when food concentrations increase (Sterner and Schulz 1998). A given increase in the limiting resource affects growth rates to a substantially smaller extent when growth is close to the maximum growth rate possible than when under conditions of low growth (low availability of a limiting resource). Because growth on unsupplemented *S. obliquus* was already close to the maximum and became maximal when EPA was supplemented, it is reasonable to assume that even smaller increases in the EPA content of *S. obliquus* would have been sufficient to increase the growth rate to  $0.5 \text{ d}^{-1}$ . However, the experiments were designed to test EPA limitation in *D. galeata* in principle; hence, I did not attempt to find the minimum increase in EPA required to achieve maximal growth rates in *D. galeata*. Despite the lack of detectable amounts of EPA in *S. obliquus*, *D. galeata* grew quite well on this alga, and EPA was only weakly limiting because EPA was endogenously synthesized from C<sub>18</sub>-PUFAs in *D. galeata*. As was shown by supplementation with SA, the EPA content of the daphnids increased with availability of higher amounts of C<sub>18</sub>-PUFAs. With the absence of EPA in the food, the animals probably become much more EPA limited when the content of C<sub>18</sub>-PUFAs in

the food is lower than in *S. obliquus*. In order to exclude the effects of food quantity in this study, growth experiments were carried out at saturating food concentrations, which resulted in the availability of high amounts of C<sub>18</sub>-PUFAs and thus promoted endogenous synthesis of EPA by the animals. In accordance with the finding that, under EPA limitation, C<sub>18</sub>-PUFAs and EPA are substitutable resources, both resources would have to be low in the natural seston for EPA to be limiting for the growth of *D. galeata* as strongly as suggested by the data from Lake Schöhsee.

In Lake Constance, correlative data indicate that  $\alpha$ -LA is limiting for *D. galeata*, whereas EPA limitation has been experimentally ruled out (Wacker and von Elert 2001). Except in the clear-water phase, EPA concentrations in Lake Constance exceeded the EPA saturation concentration calculated for Lake Schöhsee during the entire experimental period, thereby suggesting that, during EPA saturation,  $\alpha$ -LA might become limiting for the growth of *D. galeata*. Accordingly, growth on the EPA-rich *S. hantzschii* was not affected by additional EPA, but  $\alpha$ -LA was obviously limiting. Ingestion rates of *D. galeata* increase with increasing food concentrations and reach a plateau when food biomass reaches the incipient limiting level (ILL). Hence, although food concentrations were well above the ILL (for *D. galeata*, approximately  $0.5 \text{ mg C L}^{-1}$ ), ingestion rates were similar to a biomass of the diatom of  $0.5 \text{ mg C L}^{-1}$ , which translated into an availability of  $\alpha$ -LA of approximately  $3 \mu\text{g C L}^{-1}$ . Somatic growth of *D. galeata* under these conditions ( $0.38 \text{ d}^{-1}$ ) matched well the correlative saturation function found with seston from Lake Constance (Wacker and von Elert 2001). Because of the decreasing slope of saturation functions with increasing resource availability, the absolute effect on growth becomes smaller the more animals become saturated by the limiting resource. Supplementation of *S. hantzschii* with  $\alpha$ -LA led to a significant increase in growth of  $0.06 \text{ d}^{-1}$ , clearly indicating that  $\alpha$ -LA was limiting for *D. galeata*. It is reasonable to assume that the almost 20-fold increase in the amount of  $\alpha$ -LA in *S. hantzschii* after supplementation released the PUFA limitation of *D. galeata* and led a different resource to become limiting, thereby preventing a higher absolute effect of supplementation of *S. hantzschii* with  $\alpha$ -LA. Because the C:P ratios of *S. hantzschii* were close to the Redfield ratio and EPA concentrations were high, other constituents must have become limiting when EPA and  $\alpha$ -LA were available at high concentrations. Additional supplementation experiments are necessary to identify the nature of this resource.

This study provides experimental proof for particular PUFAs limiting growth of *D. galeata* when food quality is not determined by morphological, toxic, or stoichiometric constraints. Low active synthesis of PUFAs or low rates of interconversion increase the risk of zooplankton being PUFA limited. Recently, it has been speculated that the lack of these physiological capabilities in these species commonly might be caused by macronutrient limitation (Anderson and Pond 2000). This might in particular be the case for *Daphnia* with a relatively high P requirement (Sterner and Schulz 1998). DeMott (1998) presented data suggesting that PUFA-deficient diets could influence the outcome of competition between *Daphnia* species. In addition to the availability of

EPA and  $\alpha$ -LA, the animals' ability to interconvert these PUFAs might determine the competitive performance under PUFA limitation.

Correlative and experimental evidence for the role of morphology and P limitation of food particles for food quality of natural seston (DeMott et al. 2001) suggest a significance of these parameters similar to that suggested for PUFAs under natural nonlimiting phosphorus conditions. However, the small, albeit significant, growth-enhancing effects of supplementation of PUFAs in this study are most probably an underestimation of the significance of low availability of PUFAs for food quality in nature. Because of the saturating food concentrations used in this study, the limiting PUFAs or precursors of those were fairly well available to the grazers so that only moderate PUFA limitation was observed, and the net effect of supplemented EPA and  $\alpha$ -LA on growth was smaller under these almost saturating conditions than it would have been during low resource availability. Hence, the higher absolute effects of EPA or  $\alpha$ -LA supplementation should be expected with nonsaturating food concentrations and with algae with a lower content of  $\alpha$ -LA, EPA, or their precursors. In order to assess the degree of limitation in nature, carefully designed experiments are needed that use the approach presented here to manipulate directly the PUFA status of natural seston and to assess how PUFA limitation affects zooplankton in nature.

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