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Polyunsaturated fatty acids : evidence for non-substitutable biochemical resources in Daphnia galeata

POLYUNSATURATED FATTY ACIDS: EVIDENCE FOR NON-SUBSTITUTABLE BIOCHEMICAL RESOURCES IN *DAPHNIA GALEATA*

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Abstract. The factors that determine the efficiency of energy transfer in aquatic food webs have been investigated for many decades. The plant–animal interface is the most variable and least predictable of all levels in the food web. In order to study determinants of food quality in a large lake and to test the recently proposed central importance of the long-chained eicosapentaenoic acid (EPA) at the pelagic producer–grazer interface, we tested the importance of polyunsaturated fatty acids (PUFAs) at the pelagic producer–consumer interface by correlating sestonic food parameters with somatic growth rates of a clone of *Daphnia galeata*. *Daphnia* growth rates were obtained from standardized laboratory experiments spanning one season with *Daphnia* feeding on natural seston from Lake Constance, a large pre-alpine lake. Somatic growth rates were fitted to sestonic parameters by using a saturation function. A moderate amount of variation was explained when the model included the elemental parameters carbon ($r^2 = 0.6$) and nitrogen ($r^2 = 0.71$). A tighter fit was obtained when sestonic phosphorus was incorporated ($r^2 = 0.86$). The nonlinear regression with EPA was relatively weak ($r^2 = 0.77$), whereas the highest degree of variance was explained by three C_{18}-PUFAs. The best ($r^2 = 0.95$), and only significant, correlation of *Daphnia*'s growth was found with the C_{18}-PUFA α-linolenic acid (α-LA; C18:3n–3). This correlation was weakest in late August when C:P values increased to 300, suggesting that mineral and PUFA-limitation of *Daphnia*'s growth changed seasonally. Sestonic phosphorus and some PUFAs showed not only tight correlations with growth, but also with sestonic α-LA content. We computed Monte Carlo simulations to test whether the observed effects of α-LA on growth could be accounted for by EPA, phosphorus, or one of the two C_{18}-PUFAs, stearidonic acid (C18:4n–3) and linoleic acid (C18:2n–6). With >99 % probability, the correlation of growth with α-LA could not be explained by any of these parameters.

In order to test for EPA limitation of *Daphnia*'s growth, in parallel with experiments on pure seston, growth was determined on seston supplemented with chemostat-grown, P-limited *Stephanodiscus hantzschii,* which is rich in EPA. Although supplementation increased the EPA content 80–800×, no significant changes in the nonlinear regression of the growth rates with α-LA were found, indicating that growth of *Daphnia* on pure seston was not EPA limited. This indicates that the two fatty acids, EPA and α-LA, were not mutually substitutable biochemical resources and points to different physiological functions of these two PUFAs. These results support the PUFA-limitation hypothesis for sestonic C:P < 300 but are contrary to the hypothesis of a general importance of EPA, since no evidence for EPA limitation was found. It is suggested that the resource ratios of EPA and α-LA rather than the absolute concentrations determine which of the two resources is limiting growth.

Key words: alga; consumer; Daphnia; *fatty acid; food quality; grazer; herbivore; Lake Constance, European Alps; PUFA; seston.*

Introduction

A key process to be understood in the study of aquatic food webs is the regulation of the efficiency of energy transfer. In their most simple form, foodwebs can be envisioned as the use of solar energy for primary production by cyanobacteria and algae and the subsequent consumption of this biomass by primary con-

sumers (herbivores) and ultimately by predators. While primary production is most frequently limited by the availability of inorganic nutrients (mainly phosphorus and nitrogen) and occasionally light, the transfer of energy across the plant–herbivore interface is highly variable and the mechanisms determining the efficiency of energy assimilation in herbivores are poorly understood (Brett and Müller-Navarra 1997). Because the study of foodwebs was conceptualized to describe the flow of energy, and since most of the nutritional energy is contained in carbon bonds, early research on zooplankton feeding ecology focused on carbon as a mea-

sure of food availability (Lampert 1977a). However, it has been clear for many years that variation in the carbon transfer efficiency from primary to secondary production is quite large (Straile 1997). This variation can be attributed to variation in food quality, but the determinants of food quality may be of different nature.

Nitrogen and phosphorus have been suggested as determinants of algal food quality for herbivorous zooplankton. These hypotheses have mainly been supported by laboratory experiments with cultured algae (Checkley 1980, Kiorboe 1985, Giani 1991, for N-limitation; Sommer 1992, Urabe and Watanabe 1992, Sterner 1993, Sterner et al. 1993, Sterner and Hessen 1994, for P-limitation). The mineral limitation hypothesis is based on the finding that herbivorous zooplankton like *Daphnia* maintain a relatively constant C:P stoichiometry (they are homeostatic consumers) despite considerable variation in the C:P ratios of their diets (Hessen 1990, Andersen and Hessen 1991, Hessen and Lyche 1991). Direct evidence for P limitation of *Daphnia* in laboratory experiments has only recently been presented by Urabe et al. (1997), demonstrating that daily supplements of inorganic P improved *Daphnia*'s growth on a P-deficient alga and by DeMott (1998) showing that in five *Daphnia* species, growth on P-limited *Scenedesmus* was significantly increased when the P-limited alga was subjected to short term addition of P prior to being fed to the grazers. Evidence for P limitation of *Daphnia* in nature is indirect and based on C:P ratios of lake seston (Hessen 1992, Elser and Hassett 1994) showing that these natural resource C:P ratios are frequently above the C:P ratio for P limitation deduced from laboratory experiments. Furthermore, poor ingestibility or digestibility of the carbon itself may cause low carbon transfer efficiency. Increased resistance to digestion has been linked to P limitation (Van Donk and Hessen 1993, Van Donk et al. 1997) by the observation that P-limited green algae showed reduced digestibility by *Daphnia* due to thickened cell walls. Both poor ingestion or digestion may lead to a limitation which is not due to a specific nutritive compound but due to low energy though this has recently been questioned by DeMott (1998).

Another reason for low food quality may be a shortage of essential biochemicals in the diet, because such nutrients can not be synthesized by a consumer. Evidence for a dietary deficiency in essential nutrients often comes from experiments in which poor growth at high carbon concentrations is not accounted for by low ingestion, reduced digestibility, or toxin content. Lampert (1977b, c) found high rates for carbon assimilation by *Daphnia pulicaria* feeding on *Synechococcus*, but no somatic growth on a pure diet of this cyanobacterium. Additional experiments (Lampert 1981) ruled out toxicity indicating that the low quality of carbon probably was due to a nutritional deficiency in an essential compound. *Synechococcus* contains only traces of long-chained ($>C_{18}$) polyunsaturated fatty ac-

ids (two or more unsaturated bonds, PUFAs) and DeMott and Müller-Navarra (1997) recently showed that growth and reproduction of *Daphnia* improved when *Synechococcus* was supplemented with PUFA-rich fish oil emulsion. Hence, the low-carbon transfer efficiency at the *Synechococcus–Daphnia pulicaria* interface is probably due to a deficiency in long-chained PUFAs. The importance of dietary fatty acids for cladocerans is further indicated by several studies. *Moina macropora* has been shown to require fatty acids in its diet (Conklin and Provasoli 1977) and to grow better on an artificial diet based on the fatty acids of (long-chained PUFA-containing) diatoms, cryptomonads, and green algae than on a diet that mimicked the fatty acid profile of (low-PUFA) cyanobacteria (D'Abramo 1979). Evidences for low growth rates and survivorship of marine animals like fish larvae (e.g., Dendrinos and Thorpe 1987, Walford and Lam 1987), mollusks (e.g., Albentosa et al. 1993, Enright et al. 1996), and crustaceans (Kanazawa et al. 1977, D'Abramo and Sheen 1993) on diets lacking the long-chained PUFAs EPA (C20:5n–3) and docosahexaenoic acid (DHA; C22:6n–3) support the potentially limiting role of PUFAs in freshwater.

The case of the importance of PUFAs in freshwater zooplankton nutrition has recently been articulated (Brett and Müller-Navarra 1997). Evidences from experiments with monoalgal food suggest that high food quality correlates with high long-chained PUFA content. Generally, taxa like cryptophytes and chrysophytes (including diatoms) are rich in long-chained PUFAs whereas cyanobacteria are a poor PUFA source (Ahlgren et al. 1992). However, in nature many phytoplankton taxa co-occur and the diets of suspension feeding zooplankton are complex mixtures of phytoplankton, protozoa, bacteria, and detritus. *Daphnia* feed nonselectively over a broad range of particle sizes and are unable to handle and reject poor-quality particles individually (e.g., DeMott 1995). Hence, biochemically different phytoplankton taxa might serve as complementary resources as has recently been demonstrated for nutritional phosphorus (DeMott 1998) and this might cast doubts on the relevance of correlative evidences from monoalgal experiments for zooplankton nutrition in nature. The aim of the present study therefore was to investigate natural lake seston. The notion of complementary resources is implicit in the measurement of sestonic parameters and the overall effect of specific resources on growth might be deduced from correlations of determined growth rates with sestonic parameters.

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 the case of a mesotrophic lake (Müller-Navarra 1995a) and of a hypereutrophic pond (Müller-Navarra et al. 2000), EPA (C20:5n-3) showed the highest correlation with

growth of *Daphnia* which has been interpreted as a limitation by EPA and the authors hypothesized that EPA is of general importance for the trophic transfer within aquatic food webs. In this study we test for the general importance of EPA directly by determining growth rates of *Daphnia* raised on natural lake seston and on seston supplemented with an EPA-rich algal culture. Growth rates obtained on natural seston were correlated with sestonic parameters. We hypothesized that the parameter showing the tightest correlation with growth indicated limitation by this parameter and tested for this by assuming that this observed correlation should not change when we supplemented seston with EPA-rich *Stephanodiscus hantzschii.* Though we determined instantaneous somatic juvenile growth rates, the results apply as well for the intrinsic rate of population increase (*r*) which is often used as a measure of fitness (Stearns 1992) since juvenile growth can serve as a relative measure of fitness in *Daphnia* (Lampert and Trubetskova 1996).

STUDY SITE AND METHODS

Study site

Our study was carried out in 1997 in Lake Constance, a mesotrophic warm-monomictic lake at the northern fringe of the European Alps. This lake consists of two basins: the upper basin (area, 472 km; mean depth, 101 m; maximum depth, 253 m) and the lower, more eutrophic basin (area, 62 km; mean depth, 13 m; maximum depth, 40 m; Wessels 1998). The pelagic sampling site (total depth, 60 m) for the present study was located within the northwestern part of the lake, the fjordlike ''Überlinger See'' being 7 km closer to the institute than the site used in other studies (Gaedke 1998). Water was collected daily with a Ruttner Sampler from a depth of 6 m (thermocline at 15–20 m) and immediately filtered through a sieve (<30 μm) and used for laboratory growth experiments.

Laboratory growth experiments were performed from May to October 1997 by providing a clone of *Daphnia galeata* SARS, which was originally isolated from Lake Constance (Stich and Lampert 1984), with natural lake water (filtered through a <30-μm sieve), which represents the edible fraction for *Daphnia* (Burns 1968). Mothers were grown in temperature-controlled (20°C) flow-through chambers (250 mL) which were continuously supplied with a suspension of *Scenedesmus acutus* MEYEN (obtained from the Max Planck Institute for Limnology [Plön, Germany] at a nonlimiting concentration (2 mg C/L; flow rate, 60 mL/ h). *S. acutus* was grown in chemostats at a dilution rate of 0.5/d at 20°C (light, 120 μmol photons·m^{-2}·s^{-1}) in modified WC medium (Guillard 1975). For supplementation of lake seston, *Stephanodiscus hantzschii,* which had been isolated from Lake Constance, was grown in chemostats at 16°C (dilution rate, 0.25/d; light, 80 μmol photons·m^{-2}·s^{-1}) in WC medium with vitamins and with reduced phosphorus (2.5 μmol/L). Chemostat-grown cells of *S. acutus* and *S. hantzschii* were concentrated by centrifugation and resuspended in filtered lake water (0.45 μm pore-sized membrane filter). Carbon concentrations of these stock solutions of algae were estimated from photometric light extinction (800 nm) using carbon extinction equations.

To initiate an experiment, *D. galeata* was cultured for at least two generations in the flow-through system under nonlimiting food conditions. Thirty egg-bearing animals were placed into a flow-through system and neonates, released from the third clutch within 4–8 h, were subsequently fed with *S. acutus* for 2 d (2 mg C/ L). Then the animals were transferred to the growth experiments which were carried out in flow-through chambers for 4 d with food suspensions being renewed daily. Experimental food was either natural lake seston <30 μm, supplemented seston, or *S. acutus*. Each treatment consisted of three replicates with ten animals each. Somatic growth rates (*g*, d^{-1}) were calculated from the equation

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

where W_0 is the mean individual dry mass of a subsample of the animals at the beginning of the growth experiment and W_t is the mean individual dry mass of the experimental daphnids after the experimental duration of $t = 4$ d. Dry masses were mean values of at least 10 individuals, weighed on an electronic balance (Mettler UMT 2; Mettler Toledo, Im Langacher, Switzerland) which recorded to the nearest 0.1 μg. Growth rates were calculated as means for each treatment.

Nine experiments with natural seston of the upper basin of Lake Constance were run from May until October 1997. In late August, one additional experiment with natural seston from the lower, more eutrophic part of the lake was carried out. Growth rates of *D. galeata* feeding on various concentrations of the standard green algae *S. acutus* were determined in exactly the same manner to obtain the standard response curve.

Particulate parameters.—Aliquots of the sestonic food were filtered onto precombusted glass fiber filters (Whatman GF/F, 25 mm diameter; Whatman, Clifton, New Jersey, USA), dried, and analyzed for particulate organic carbon (POC) and particulate organic nitrogen (PON) (NCS-2500 analyzer, ThermoQuest GmbH, Egelsbach, Germany).

For determination of particulate phosphorus (P$_{part}$), aliquots of the sestonic food were collected on acid-rinsed polysulfon filters (HT-200; Gelman Sciences, Ann Arbor, Michigan, USA) and digested with a solution of 10% potassium peroxodisulfate and 1.5% sodium hydroxide in an autoclave for 60 min; soluble reactive phosphorus (SRP) was determined using the molybdate-ascorbic acid method (Greenberg et al. 1985).

Fatty acid analysis

Aliquots of natural lake water filtered through a <30 μm sieve representing ∼1.0 mg POC were filtered through a precombusted GF/F filter (Whatman, 47 mm diameter). The loaded filters were either extracted immediately or stored at −20°C for later analysis. Lipids were extracted with dichlormethane/methanol (2:1, v/v) and transesterified with 3 M methanolic HCl (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) according to Mason and Walker (1964) with heptadecanoic acid methylester and tricosanoic acid methyl ester as internal standards. Details are given elsewhere (Von Elert and Stampfl 2000). Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography using a gas chromatograph (CE-Fractovap Series 2150; ThermoQuest GmbH, Deisenhofen, Germany) with the following configuration: column, DB 225 (J&W Scientific, Köln, Germany; 30 m × 0.25 mm ID, 0.25 μm film); oven, 140°C (3 min) to 210°C at 2°C/min, hold for 30 min; carrier, Helium 26 cm/sec; detector, FID 250°C; injector, 250°C, total run time 55 min/sample. A 1-μL aliquot of the sample was injected splitlessly. FAMEs were identified by comparison of retention times with those of reference compounds and by GC-MS (Finnigan GCQ; ThermoQuest GmbH). Quantification was performed by referring to the internal standards and to response factors determined for each FAME from mixtures of known composition. The detection limit was 40 ng/mg C and it was not possible to distinguish between petroselinic acid (C18:1n–12) and oleic acid (C18:1n–9). The absolute amounts of each FAME were related to the independently determined POC content of the sample. Reference FAMEs were purchased from Sigma-Aldrich Chemie GmbH.

For each of the nine growth experiments, two single fatty acid analyses (day 1 and day 3) were carried out and averaged so that variation of the fatty acid data can not be assigned directly. Replicate analyses of seston samples ($n = 3$) showed that for each of the fatty acids the coefficient of variance (CV = SD·100/mean) ranged from 4% to 6% (4.8% for α-LA). The function which correlated growth rates with sestonic α-LA was used in order to estimate the SD that would be due to variation in the fatty acid values. A CV of 4.8% for each of the nine means of sestonic α-LA translated into changes in growth rates that always were smaller than the SD obtained from the growth experiments and on average were 62% of the SD calculated from the experiments.

Data analysis

All determined seston parameters were correlated with the somatic growth rates, *g*, of *D. galeata* fed on this seston. For *D. galeata* feeding on a standardized food (chemostat-grown *S. acutus*), growth rates as a function of the food-constituent carbon could be fitted very well by a modified Monod model (Fig. 1). Hence
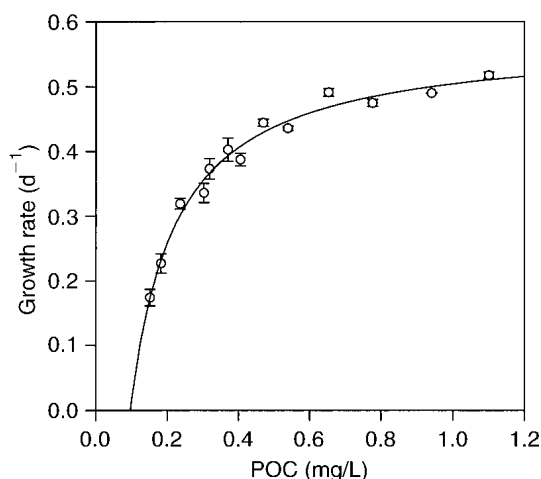

FIG. 1. Growth response curve of *Daphnia galeata* feeding on *Scenedesmus acutus*. The regression line was calculated according to a modified Monod model. Values are means ± 1 SD. POC is particulate organic carbon.

the functional relationships between sestonic concentrations and growth rates were expressed as Monod curves (Monod 1950) modified with a threshold S_0 for zero somatic growth

$$g = g_{max}\left[\frac{c - S_0}{c - S_0 + K_S}\right]$$

where g_{max} = maximum growth rate (d^{-1}), c = resource concentration (μg/L), S_0 = threshold concentration for zero growth (μg/L), K_S = half saturation constant (μg/L). The curves were fitted to data by iterative nonlinear regression (Statistica 4.5; StatSoft, Inc., Tulsa, Oklahoma, USA). R^2 and standard deviations from nonlinear regression are an approximate guide to uncertainties in parameter estimates. Probabilities of type I error of parameter estimates can be used to test the quality of the nonlinear regression. Out of the three parameter estimates, the half saturation constant, K_S, showed highest values of probability. Therefore, this constant, K_S, was the strongest part of the uncertainty, and we used this parameter for describing the nonlinear regressions quality. The significance of K_S was determined after sequential Bonferroni adjustment (Rice 1989).

We conducted Monte Carlo simulations (Day and Quinn 1989) of the affiliation of several sestonic parameters to the observed correlation of growth rates with α-LA. This was done by applying the intercorrelation of a particular parameter with α-LA in order to randomly generate 100 values for α-LA for each of nine values determined for a particular sestonic parameter with 95% of the generated values being within the 95% confidence limits of the intercorrelation. On the basis of these generated values of α-LA, nonlinear regressions of growth rates were calculated 100 times and the r^2 values of these simulations were compared with r^2 values obtained for the regression of growth rates as a function of measured values of α-LA. All

data were generated using the random number generator routine of Statistica 4.5.

We examined whether supplementation of pure seston with *S. hantzschii* was reflected in changes of the nonlinear fit of the Monod model by using a nonlinear analog to ANCOVA (Ratkowsky 1983). For each of both growth response curves (seston and supplemented seston) the sum-of-squares for error (SSE) of the Monod model was determined. The SSE from each of the individual data sets were added to produce a pooled SSE. Additionally, the SSE from a common model including the data from both treatments was computed. The significance of differences in the SSE from the common and the two individual models were then assessed by an *F* test.

From the pooled data set of the experiments with pure and supplemented seston, a threshold for zero growth, S_0, was calculated from the nonlinear regression of growth rates vs. the sestonic content of α-LA. In the experiments with supplemented seston, supplementation resulted in too few experiments with low concentrations of α-LA, so that a value for S_0 could not be calculated from the nonlinear regression. Since the threshold S_0 of a limiting resource can be assumed to remain unaffected from changes in the availability of other resources, the value for S_0 calculated from the pooled data was applied to the nonlinear regression for supplemented seston.

RESULTS

The concentration of particulate organic carbon (POC) <30 μm in the lake varied from 0.6 mg C/L in spring to a minimum of 0.2 mg C/L in the clear water phase and to a maximum of 0.8 mg C/L during the summer bloom. The seasonal changes of particulate phosphorus (P_{part}) were similar, but P_{part} increased considerably less than POC after the clear water phase leading to a maximum of the molar C:P ratios of 300 in late August, whereas it was <200 during the remainder of the season (Fig. 2A). The somatic growth rates of *Daphnia galeata* raised on natural seston showed a pronounced seasonality with a maximum of 0.56/d in spring and a minimum of 0.2/d during the clear water phase (Fig. 2B). From July to September only moderate growth rates of 0.3–0.4/d were observed.

The growth rates of *D. galeata* as a function of sestonic POC <30 μm showed considerable scatter (Fig. 3A) and the nonlinear correlation was weaker ($r^2 = 0.6$, Table 1) than the correlation for animals raised on *Scenedesmus acutus* (Fig. 1). POC, varying from 0.2 to 0.8 mg C/L, was temporarily above the incipient limiting level reported for cultured algae of high food quality (McMahon and Rigler 1965, Lampert 1978). The correlations with sestonic phosphorus and nitrogen (Fig. 3B, C) were stronger than for carbon (Fig. 3A), but were not significant (Table 1).

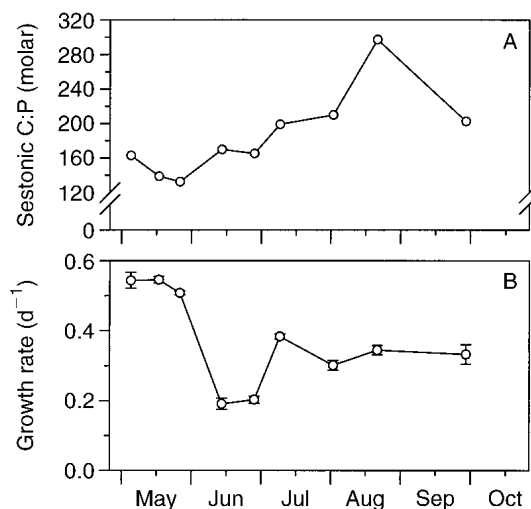When, instead of stoichiometric parameters, total

FIG. 2. (A) Seasonal pattern of sestonic molar C:P ratios and (B) somatic growth rates of *Daphnia galeata* raised on seston. Data refer to the edible (<30 μm) fraction of natural seston. Values are means ± 1 SD.

sestonic fatty acids as a summarizing parameter were used for the nonlinear regression analysis, the correlation was poor ($r^2 = 0.70$; Fig. 3D, Table 1). The sum of (n-3) and (n-6) polyunsaturated ≥C18 fatty acids (long-chained PUFAs) correlated as well as sestonic phosphorus with growth rates but was not significant (Table 1). When instead of total fatty acids, individual fatty acids were used for the nonlinear regression, eicosapentaenoic acid (EPA, C20:5n–3; $r^2 = 0.77$; Fig. 4A, Table 1) and all other long-chained PUFAs led to a poorer correlation than sestonic nitrogen and phosphorus. However, almost all of the variation in the data was explained by each of the three C18- PUFAs linoleic acid (C18:2n–6), stearidonic acid (C18:4n–3), and α-linolenic acid (C18:3n-3), with the latter being the only parameter correlating significantly (Table 1, Fig. 4B–D). Hence the sestonic α-linolenic acid (α-LA) concentration was a very good predictor for daphnids' growth during the whole period studied. In the nonlinear regression of somatic growth rates with α-LA, one point attracts attention where growth rates were lower than predicted by the regression line (Fig. 4B). This data point refers to an experiment carried out in late August when seston showed an exceptionally high molar C:P ratio (300:1), while in all other cases C:P ratios ranged from 130 to 200.

Twenty-four different seston parameters were correlated with growth rates (Table 1). Hence, we could not exclude the possibility that, even after sequential Bonferroni adjustment, the observed significance of the correlation with α-LA was accidental. Furthermore, fatty acid profiles of algae might vary with nutrient limitation (Parrish et al. 1991, Müller-Navarra 1995*b*) and many of the sestonic parameters were highly correlated with α-LA (Table 2). Sestonic P content has been shown and EPA is discussed to determine food
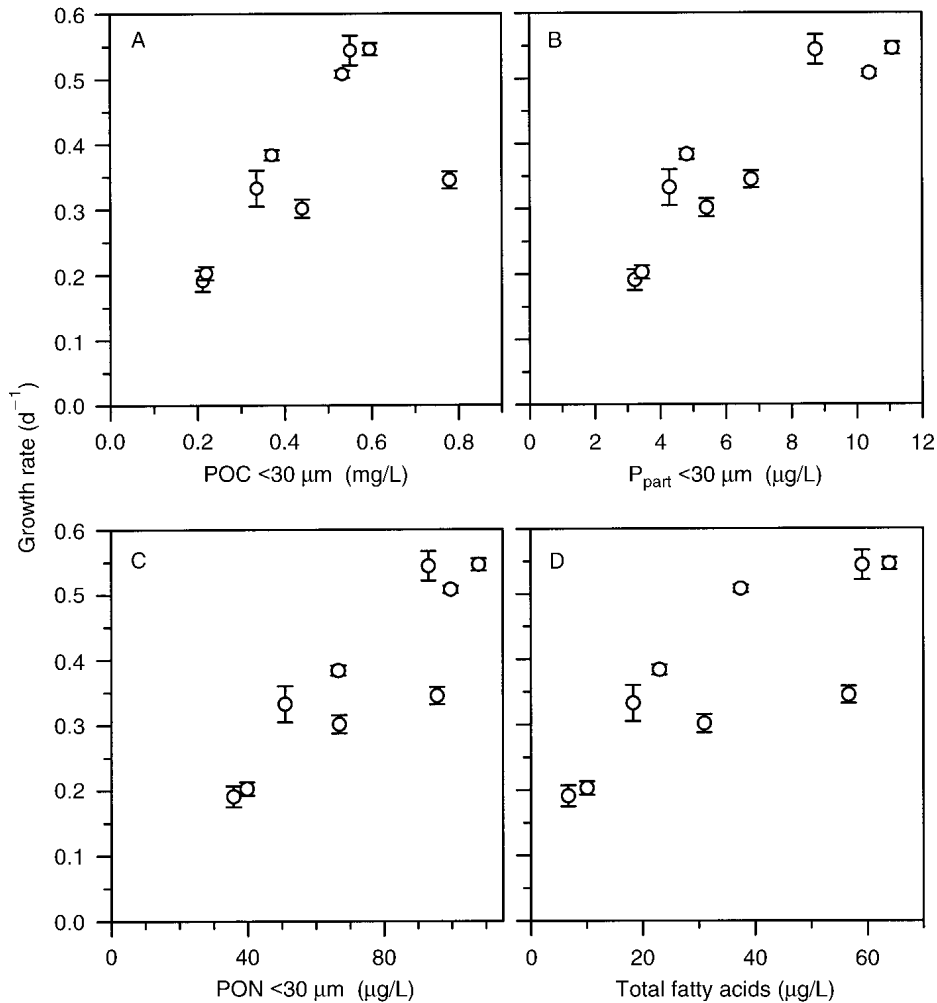
FIG. 3. Somatic growth rates of *Daphnia galeata* as a function of the sestonic food parameters: (A) particulate organic carbon (POC), (B) particulate phosphorus (P_{part}), (C) particulate organic nitrogen (PON), and (D) total fatty acids from the edible (<30 μm) fraction. Data points represent independent experiments performed at different dates of the season. Data are means ± 1 SD for three replicates.

quality (reviewed by Sterner and Schulz 1998). This led to the hypotheses that the significant correlation observed for α-LA can be explained by the sestonic content of EPA or P. In order to calculate probability values for these hypotheses we conducted Monte Carlo simulations of sestonic α-LA.

In the case of sestonic phosphorus (P) the hypothesis was that the observed significant effect of α-LA on growth can be explained by the P data. We therefore plotted the sestonic α-LA values referring to the nine independent experiments vs. the nine sestonic P values and determined the regression for α-LA as a function of P. Assuming that P determines the α-LA values, we generated 100 α-LA values for each of the nine P values by using the above-determined regression for α-LA as a function of P so that we created nine "clouds," each comprising 100 α-LA values. Ninety-five percent of the generated values were within the 95% confidence limits of the above-determined regression for α-LA as a function of P. Then, for each of the nine P values,

we randomly picked one out of the referring hundred values and with these (generated) nine values of α-LA we calculated a nonlinear regression of growth rates vs. these randomly picked nine α-LA values. We performed this procedure of randomly picking α-LA values and correlating the growth rates vs. these α-LA values a hundred times so that we ended up with 100 values of r^2. The maximum value of r^2 obtained from the simulation was 0.928 (Fig. 5). Hence, none of these 100 values of r^2 was equal or higher than the one calculated from sestonic α-LA ($r^2 = 0.953$; Fig. 5) so that it is rather unlikely (<1% probability) that the correlation observed for α-LA can be explained by P.

We used the same approach to generate probability values for the hypothesis that sestonic EPA (C20:5n–3) explains the correlation observed for α-LA. Using α-LA values that had been generated on the basis of the intercorrelation with EPA for correlation with growth rates resulted in 100 values of r^2 which all were <0.915 (Fig. 5), so that an explanation of the effect of

TABLE 1. Correlations of somatic growth rates of *Daphnia galeata* raised on natural seston with concentrations of sestonic parameters with a modified Monod model ($n = 27$): $g = g_{max}(c - S_0)/(c - S_0 + K_s)$.

Parameters	r^2	$P(K_s)$
POC	0.60	0.20
PON	0.71	0.47
P_{part}	0.86	0.31
C14:0	0.57	0.14
C15:0	0.52	0.20
C16:0	0.75	0.24
C16:1	0.44	0.14
C18:0	0.66	0.49
C18:1n–12/n–9	0.64	0.07
C18:1n–7	0.80	0.37
C18:2n–6	0.89	0.70
C18:3n–6	0.33	0.27
C18:3n–3	0.95	0.0001†
C18:4n–3	0.91	0.018
C20:0	0.43	0.20
C20:1n–9	0.03	0.8
C20:4n–6	0.70	0.2
C20:5n–3	0.77	0.18
C22:0	0.49	0.10
C22:6n–3	0.59	0.16
C24:0	0.28	0.14
Total fatty acids	0.70	0.29
Sum of all n–3	0.86	0.12
Sum of all n–6	0.86	0.13

Note: The proportion of variance accounted for (r^2) and P values for the half-saturation constant K_s are depicted.

† Significant after Bonferroni adjustment ($P < 0.002$).

α-LA on growth rates of *D. galeata* by EPA could be rejected on a 99% probability level. Thus, there is indeed an effect of α-LA on growth rates which can not be explained by sestonic EPA or P content.

However, rejecting effects of the food quality parameters P and EPA which have been shown to be important in other lakes still leaves the possibility that parameters highly intercorrelating with α-LA in Lake Constance can explain the observed high predictive power of α-LA. The highest intercorrelations with α-LA were found for the C18-PUFAs C18:4n–3 and C18:2n–6 (Table 2). We hypothesized that one of these two C18-PUFAs determined the α-LA concentrations. Using α-LA values, that had been generated by Monte Carlo simulations on the basis of the intercorrelation with C18:4n–3 and C18:2n–6, for correlation with growth rates led to 100 values of r^2 which all were <0.93 for C18:4n–3 and <0.945 for C18:2n–6. Hence, none of these 100 values of r^2 was equal or higher than the one calculated from sestonic α-LA ($r^2 = 0.953$; Fig. 5) so that it is rather unlikely (<1% probability) that the correlation observed for α-LA can be explained by either of these two C18-PUFAs.

The long-chained PUFA EPA has recently been suggested to be of general importance in freshwater zooplankton limitation (Müller-Navarra et al. 2000). We wanted to experimentally test for the putative limitation of *D. galeata* by sestonic EPA by supplementing seston with a standardized alga rich in EPA. In parallel with the nine experiments on pure seston <30 μm, we ran nine growth experiments with seston that was supplemented with chemostat-grown P-limited *Stephanodiscus hantzschii,* which was rich in EPA (Table 3) and poor in P (molar C/P = 386). Since the C:P ratio of *S. hantzschii* exceeded the values observed for seston (Fig. 1) no increase of the sestonic P content was to be expected from supplementation. The increase of POC due to supplementation in all nine experiments was on average 28% whereas EPA concentrations increased in the range from 80% to 800% (Table 4). Except for the two early spring experiments, supplementation led to a significant increase of *Daphnia*'s growth in each of the remaining seven experiments (a mean increase of 31%, range 7–76%) indicating that *S. hantzschii* was assimilated by the grazer. If the growth of *Daphnia* indeed was limited by α-LA then, after supplementation, growth rates should again be strongly correlated with the α-LA content, regardless of the substantially increased EPA-concentrations (Fig. 6B, case II). Unless growth was limited by α-LA, supplementation with *S. hantzschii* might lead to different effects on growth. If growth is limited by a resource which is a constituent of *S. hantzschii* then growth observed after supplementation should be higher than predicted by the α-LA content of supplemented seston (Fig. 6B, case III), or, if supplementation shifts the limitation by α-LA to limitation by any other resource, then growth observed after supplementation should be lower than predicted by the α-LA content of supplemented seston (Fig. 6B, case I). However, both cases would be reflected in changes of the nonlinear fit of growth rates as a function of sestonic α-LA. The significance of changes was tested using a nonlinear analog to ANCOVA (Ratkowsky 1983). No significant changes in the nonlinear regression of the growth rates with α-LA by adding *S. hantzschii* to the seston were found ($F_{2,50} = 2.9$, MSE = 0.0012, $P > 0.05$) indicating that growth of *Daphnia* on pure seston was not EPA limited. The finding of no significant changes supports the hypothesis of a common regression of growth as a function of α-LA for both pure and supplemented seston and hence corroborates the correlative evidences for growth limitation by α-LA.

The predictive power of α-LA for growth of *Daphnia* was given further support when a single growth experiment was carried out with seston from the lower basin of Lake Constance, a more shallow and eutrophic part. The lower basin's seston fitted well into the correlation of α-LA with growth rate established for the upper basin's seston (Fig. 4B): If the lower basin's data were included in the correlation of the upper basin the equation would stay the same, which supports the predictive power of α-LA for growth of *D. galeata*.

DISCUSSION

The concept of food quality implies that not all units of food measured in carbon will be equal from a nutritional point of view. Animals obtain a large set of
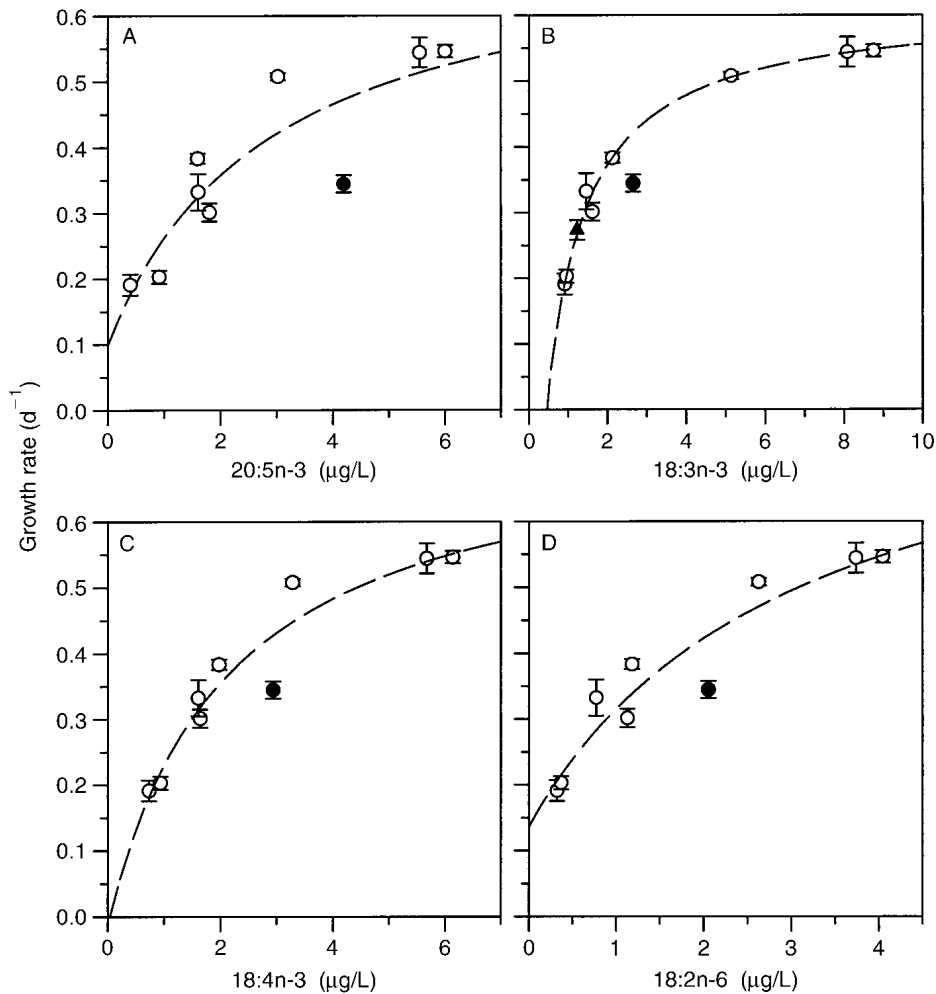
Fig. 4. Somatic growth rates of *Daphnia galeata* as a function of the sestonic (<30 μm) content of (A) eicosapentaenoic acid (C20:5n–3), (B) α-linolenic acid (C18:3n–3), (C) stearidonic acid (C18:4n–3), and (D) linoleic acid (C18:2n–6). Data points represent independent experiments performed at different dates of the season. Data are means ± 1 SD for three replicates. Regression lines were calculated according to a modified Monod model. In (B), all data points of the upper basin (open circles) are included in the regression, with filled circles referring to seston with a molar C:P of 300:1. Closed triangles refer to an independent experiment with seston from the lower, more eutrophic basin of Lake Constance, which was not included in the Monod model.

essential or nearly essential compounds from their food. Besides the elements there are numerous biochemicals that can not be synthesized at all or only at rates too slow to meet the demands and hence determine the quality of the units of carbon of the food. Biochemicals and elements vary and covary with growth conditions in cultured algae (Kilham et al. 1997a) and in lakes (Kreeger et al. 1997). In addition to these variations in determinants of quality the level of particulate organic carbon (POC) fluctuates under natural conditions during the season so that quantitative and qualitative food limitation might alternate which complicates the identification of a limiting dietary compound in field studies. The approach used here consists of an indirect (correlations) and a direct (supplementations) one. In the indirect approach the concentration of a food constituent of natural seston is correlated to

somatic growth of *Daphnia*. A high correlation provides strong evidence for a food quality limitation by this constituent. In the direct approach the kind of qualitative limitation is tested by supplementing the seston with a standardized cultured algae and thereby increasing the concentration of a putatively limiting resource.

Elements, of course, if needed, are essential as they cannot be synthesized from other elements. Most of zooplankton studies on elemental limitation have focused on the role of phosphorus and studies indicate that daphnids are limited by phosphorus above a C:P ratio threshold of 300 (Urabe and Watanabe 1992, Sterner 1993, Urabe and Sterner 1996). This agrees with our observation that the predictive power of the sestonic α-linolenic acid (α-LA; C18:3n–3) content for growth was weakest in late August when sestonic C:P values increased to 300. *Daphnia* growth experiments

TABLE 2. Correlation of sestonic parameters with sestonic α-linolenic acid content.

Parameters	r^2	P
POC	0.34	<0.01
PON	0.68	<0.01
P_{part}	0.82	<0.001
C14:0	0.31	<0.01
C15:0	0.31	<0.01
C16:0	0.74	<0.01
C16:1	0.21	<0.05
C18:0	0.59	<0.001
C18:ln–12/n–9	0.14	0.06
C18:ln–7	0.91	<0.001
C18:2n–6	0.96	<0.001
C18:3n–6	<0.01	0.75
C18:4n–3	0.97	<0.001
C20:0	0.16	<0.05
C20:ln–9	<0.01	0.98
C20:4n–6	0.67	<0.001
C20:5n–3	0.84	<0.001
C22:0	0.02	0.52
C22:6n–3	0.47	<0.001
C24:0	0.02	0.45

Note: The proportion of variance accounted for (r^2) and P values are shown.

with mixtures of P-limited and P-replete algae have provided strong support for the hypothesis that the reason for low quality of high C:P algae is indeed P limitation of *Daphnia* (DeMott 1998). Here we report sestonic C:P values in the range from 130 to 300 for the whole season and only a nonsignificant correlation of
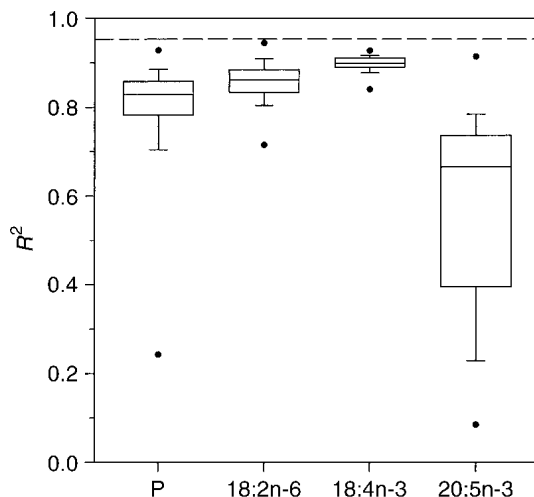

FIG. 5. Results of the Monte Carlo simulations of sestonic α-linolenic acid (C18:3n–3) based on the measured concentrations of particulate phosphorus (P), linoleic acid (C18:2n–6), stearidonic acid (C18:4n–3), and eicosapentaenoic acid (C20:5n–3). R^2 values resulting from nonlinear regressions of somatic growth rates of *Daphnia galeata* vs. simulated concentrations of α-linolenic acid are given as median and 25th/75th percentile (box) and 10th/90th percentile (whisker) and as minimum and maximum (filled circles). All R^2 values derived from the simulated α-linolenic acid concentrations were smaller than the one for the nonlinear correlation of growth rates with measured α-linolenic acid concentrations (---).

TABLE 3. Fatty acid content of the chemostat-grown phosphorus-limited *Stephanodiscus hantzschii* used for the supplementation of seston <30 μm.

Fatty acid	Concentration (μg/mg C)†	Relative abundance (%)‡
C14:0	36.52	12
C15:0	1.11	0.36
C16:0	41.74	13.7
C16:1	138.47	45.5
C18:0	5.59	1.84
C18:ln–12/n–9	3.11	1.02
C18:ln–7	2.23	0.73
C18:2n–6	3.58	1.18
C18:3n–6	0.90	0.29
C18:3n–3	2.74	0.9
C18:4n–3	7.64	2.51
C20:0	0.42	0.14
C20:3n–6	0.35	0.12
C20:4n–6	1.08	0.35
C20:5n–3	45.09	14.8
C22:0	0.50	0.17
C22:ln–9	1.12	0.37
C22:2n–6	0.64	0.2
C22:6n–3	11.57	3.8
C24:0	0.14	0.05
Sum FAs	304.55	100
Sum n–3 FAs	67.05	22
Sum n–6 FAs	6.55	2.15

† Concentration normalized to POC.
‡ Relative abundance normalized to total fatty acids.

sestonic phosphorus with *Daphnia*'s growth rates. Since the composition of algal fatty acids largely varies with conditions of growth (Cobelas and Lechardo 1988, Harrison et al. 1990) and due to a high intercorrelation of α-LA and phosphorus it could not be excluded that the observed significant correlation for α-LA can be explained by phosphorus. However, based on a Monte Carlo simulation it is rather unlikely (<1% probability) that the correlation observed for α-LA can be explained by phosphorus and hence that phosphorus per se was limiting *Daphnia galeata* growth in Lake Constance. This is consistent with the general pattern that in larger lakes, due to a higher mixing depth, light is relatively less available compared to nutrients and hence the base

TABLE 4. Effect of supplementation of seston <30 μm with chemostat-grown *Stephanodiscus hantzschii*: increase of the concentrations of particulate organic carbon (POC) and of eicosapentaenoic acid (EPA) normalized to pure seston.

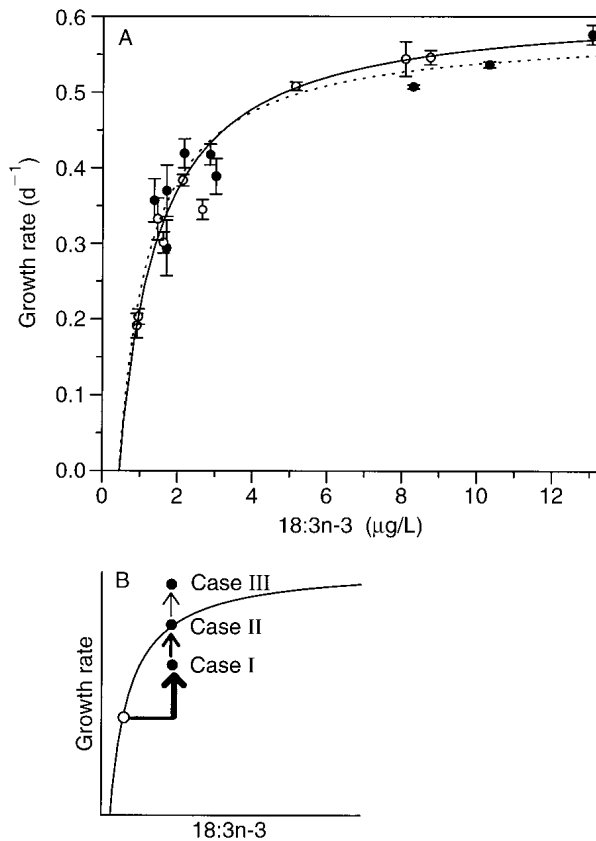| Date of experiment (1997)	Increase of POC (%)	Increase of EPA concentration (%)
5 May	25.9	109.3
17 May	18.7	78.9
26 May	18.0	224.3
13 June	19.2	808.1
27 June	54.4	426.3
8 July	26.2	147.5
31 July	22.1	159.1
19 August	14.2	105.7
26 September	55.8	355.0

FIG. 6. (A) Somatic growth rates of *Daphnia galeata* as a function of the sestonic (<30 μm) content of α-linolenic acid (α-LA, C18:3n−3) of pure seston (open circles) and of seston supplemented with EPA-rich *Stephanodiscus hantzschii* (filled circles). Values are means ± 1 SD. (B) If growth of *D. galeata* was limited by α-LA, after supplementation growth rates should still be strongly correlated with the α-LA content, regardless of the substantially increased EPA concentrations (case II). Unless growth was α-LA-limited, supplementation with *S. hantzschii* might lead to growth higher than predicted by the α-LA content of supplemented seston (case III; limitation by a resource which is a constituent of *S. hantzschii*) or to growth lower than predicted by the α-LA content of supplemented seston (case I; shift of limitation from α-LA to limitation by any other resource).

of the food web is relatively P rich (Sterner et al. 1997). Our finding that P is not determining food quality is in agreement with two studies where the somatic growth rates of *D. galeata* with respect to *D. magna* showed a weak correlation to the sestonic phosphorus concentrations and where C:P ratios were below 300 (Müller-Navarra 1995*a,* Müller-Navarra et al. 2000). These results are further supported by Hessen (1989) who reports a nonsignificant relation between sestonic organic phosphorus and *Daphnia* egg production in a humic lake over one season.

Below the C:P threshold of 300 other food ingredients become limiting for the animals (Sundbom and Vrede 1997). Such ingredients may be biochemical food constituents like particular fatty acids as can be concluded from studies with algal cultures. Ahlgren et

al. (1990), using monoalgal food, attributed the poor food quality of cyanobacteria on the one hand and the good food quality of Cryptophyceae on the other to different fatty acid compositions of the algal species. Cryptophyceae like *Cryptomonas* and *Rhodomonas* are rich in long-chained PUFAs (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) while Chlorophyceae contain high amounts of α-LA (Ahlgren et al. 1992). Ahlgren et al. (1990) therefore suggested n-3 long-chained PUFAs to be important in determining food quality for cladocerans which was confirmed by Müller-Navarra (1995*b*).

These correlative evidences with unialgal food are supported by supplementation experiments, an approach which provides the most direct experimental test of limitation by a single dietary compound. Weers and Gulati (1997) could improve the quality of the low long-chained PUFA alga *Scenedesmus* (undetectable EPA and DHA content) for *D. galeata* slightly, by adding a PUFA-rich emulsion, whereas an emulsion of more saturated and shorter fatty acids had no significant effect on food quality. Adding microencapsulated fish oil (rich in EPA and DHA) to *Scenedesmus* increased somatic growth and fecundity of *D. galeata* (Sundbom and Vrede 1997). However, when only microencapsulated EPA was added to *Scenedesmus,* somatic growth was not affected but the percentage of egg-carrying females increased. Adding droplets of fish oil, which was rich in EPA and DHA, to the cyanobacterium *Synechococcus* (only traces of EPA and no DHA) improved the somatic growth of three *Daphnia* species (DeMott and Müller-Navarra 1997) suggesting that the low quality of the cyanobacterial carbon is due to a deficiency in one or more of the fish oil constituents.

Attempts to identify biochemical limitation of zooplankton in the field are scarce. In correlative studies within a lake (Müller-Navarra 1995*a*) and within a pond (Müller-Navarra et al. 2000), EPA was the seston parameter correlating best with the growth of *Daphnia* in a standardized growth assay. In a field survey of 23 ponds and lakes De Lange and Arts (1999) found the phospholipid to be the best seston variable to explain growth of *D. magna* in a biotest. The present study comprises the first attempt to investigate food quality parameters in a large lake and to test by supplementation the recently presented hypothesis that EPA seems to be of general importance for the trophic transfer within aquatic food webs (Müller-Navarra et al. 2000). The correlation between somatic growth and particulate organic carbon (POC) was moderate but not significant indicating that food quantity was not a good predictor for growth. However, the sestonic content of α-LA was the only significantly correlating parameter and left only 5% of the variation unexplained. This extraordinarily high predictive power of α-LA for food quality is contrasted by the only modest correlation of somatic growth with EPA. Though EPA intercorrelated signif-

icantly with the sestonic content of α-LA it can be excluded on a 99% probability level that EPA explains the observed effect of α-LA on daphnids' growth in Lake Constance. These correlative evidences for the minor importance of EPA as a determinant of food quality were supported by the results from supplementation experiments: compared to experiments with pure seston the predictive power of α-LA for somatic growth of *Daphnia* remained undistinguishably high despite substantially increased EPA concentrations. Increased growth after addition of *S. hantzschii* to seston indicates that the diatom was assimilated by *Daphnia* and was probably not toxic. Though we can not exclude that a positive effect of EPA delivered with *S. hantzschii* was outweighed by another constituent of the alga, this would require the assumption that a constituent of *S. hantzschii* specifically inhibits the utilization of EPA without inhibiting the assimilation of this alga. Additional experiments (E. Von Elert, *unpublished data*) have shown that growth of *Daphnia* on *S. hantzschii* is not limited by EPA indicating that EPA delivered with the diatom was available to *Daphnia* and corroborating the conclusion that growth on seston was not EPA limited. The exceptionally strong correlation between growth rates and α-LA suggests that *Daphnia*'s somatic growth was limited by α-LA throughout almost the entire season from May until October.

Though confirming the predictability of carbon transfer between primary producers and consumers by PUFAs that has been reported by Müller-Navarra (1995*a*) for the mesotrophic Lake Schöhsee and for a hypereutrophic pond (Müller-Navarra et al. 2000), our data do not support the recent suggestion that EPA seems to be of general importance for the trophic transfer within the aquatic food web (Müller-Navarra et al. 2000). This suggestion was based on the findings that in the case of Lake Schöhsee, using a nonlinear asymptotic model, EPA correlated best with growth rates of *Daphnia*; and in the case of the pond, using a linear model, the EPA content of food carbon correlated best with growth rates of *Daphnia*. In Lake Constance, α-LA explained much more variance ($r^2 = 0.95$) in growth rates than EPA ($r^2 = 0.77$), whereas EPA was a much better predictor of somatic growth than was α-LA in Lake Schöhsee ($r^2_{EPA} = 0.93$; $r^2_{α-LA} = 0.78$) and in the hypereutrophic pond ($r^2_{EPA} = 0.95$; $r^2_{α-LA} = 0.26$). While α-LA concentrations in Lake Schöhsee (Müller-Navarra 1993) were in the same range as in Lake Constance, Müller-Navarra (1995*a*) calculated for the putatively EPA-limited seston of Lake Schöhsee an EPA-saturation concentration of 0.8 μg/L. Except for the clear-water phase (0.4 μg/L) EPA concentrations in Lake Constance exceeded this concentration during the whole experimental period so that a limitation of *D. galeata* by EPA was unlikely thus explaining the poor predictive power of EPA in Lake Constance.

It is obvious that limitation by EPA cannot be generalized for freshwater systems with C:P ratios <300, but rather represents a special case of limitation by n-3 PUFAs. We therefore hypothesize that the ratio in which PUFAs are contained in the diet determines which particular PUFA is limiting. *Daphnia* raised on a low-EPA seston will be limited by EPA like in Lake Schöhsee whereas animals raised on high-EPA seston like that in Lake Constance are limited by a different n-3 PUFA, in this case α-LA. The reasons for the obvious low substitutability of EPA and α-LA may be poor conversion rates of α-LA to EPA that have been reported for *Daphnia* (Weers et al. 1997) and which are in agreement with observed low conversion capacities of unsaturated fatty acids for other aquatic invertebrates (Kanazawa et al. 1977, Langdon and Waldock 1981, Waldock and Holland 1984). In Lake Constance, daphnids seem to be limited by α-LA though EPA is available in high concentrations. This suggests, although no direct biosynthesis work was done, that the physiological role of α-LA, albeit unknown, is distinct from the functions of EPA and that EPA can not be converted into α-LA. A similar finding is reported for the waxmoth *Galleria melonella* which requires dietary EPA and α-LA for normal development of adults (Stanley-Samuelson 1987). On a diet rich in EPA but free of α-LA, morphologically abnormal adults develop. This nutritional deficiency in α-LA can be compensated by C20:3n–3 and C22:3n–3 which are converted into α-LA, whereas EPA cannot serve as a precursor for α-LA due to the high degree of unsaturation. Hence, the two PUFAs α-LA and EPA, which are known to be essential in many animals (Stanley-Samuelson et al. 1988), seem to have different physiological functions and are not mutually replaceable, so that both compounds may be regarded as nonsubstitutable resources. Though, in lakes with sestonic C:P <300, concentrations of single PUFAs seem to be a powerful predictor of food quality within the very lake, in the light of the only three correlative field studies on PUFAs and food quality (Müller-Navarra 1995*a*, Müller-Navarra et al. 2000, this study) it is tempting to suggest that ratios, rather than concentrations, of nonsubstitutable PUFAs (e.g., EPA:α-LA) might provide a good criterion for the comparison of different lakes. Similarly Kilham et al. (1997*b*) found that ratios of protein:lipid were better predictors for *Daphnia* population growth than absolute quantities of each.

Though n-6 PUFAs are known to be essential for many animals (Stanley-Samuelson et al. 1988), none of the three correlative field studies provides evidence for a putative limitation by these compounds. It is unclear whether this is due to a sufficient supply ratio or a nonessential character of n-6 PUFAs for *Daphnia*.

Long-chained PUFAs like EPA are absent from cyanobacteria, which are reported to be low quality food for *Daphnia* due to nutritive deficiencies, toxicity, and poor ingestibility or digestibility. In any correlative study on food quality of seston in which cyanobacteria comprise a certain part of the carbon, low food quality

may to a certain degree be correlated to poor ingestibility and digestibility rather than to a nutritional deficiency. In such cases specific PUFAs, which are not present in cyanobacteria, can be assumed to correlate well with good ingestibility and digestibility and hence might be a good predictor of food quality but, for the same reason, need not be the limiting compound. This might have been the case in a hypereutrophic pond with filamentous cyanobacteria dominating in summer and diatoms in winter and with the finding that the non-cyanobacterial long-chained PUFA EPA correlated best with food quality (Müller-Navarra et al. 2000). Though the authors could rule out that poor ingestibility was the reason for low food quality during cyanobacterial dominance, low digestibility of the filamentous cyanobacteria (Porter 1977) compared to diatoms can not be excluded. Thus, there is a higher risk that a non-cyanobacterial PUFA like EPA does not represent a limiting resource for growth of *Daphnia* than with a PUFA like α-LA, that is as well present in cyanobacteria (Kenyon and Stanier 1970, Kenyon 1972). A tight correlation of a compound like α-LA with growth would certainly be weakened if low cyanobacterial digestibility would be a determinant of low carbon transfer efficiency. However, in case of the reoligotrophicating Lake Constance, cyanobacteria were definitely of minor importance (Gaedke 1998).

This study provides strong support for the hypothesis that PUFAs are a potentially limiting resource for growth of *Daphnia* in nature unless digestion resistance, deficiencies in phosphorus, or other factors strongly limit food quality. We present strong correlative evidence that EPA was not limiting *Daphnia*'s growth in Lake Constance which was experimentally confirmed by supplementation experiments with an EPA-rich alga. Instead, correlative results strongly indicate that there is an effect of α-LA on somatic growth which cannot be explained by other highly intercorrelating parameters (>99% probability). The assumption that the observed tight correlation of α-LA with somatic growth indicates limitation of *D. galeata* by this resource is supported by the supplementation experiments. Though the indication that α-LA is limiting *Daphnia*'s growth in nature suggests that α-LA is essential for daphnids, this study is confined to the limitation by PUFAs, and the essential character of PUFAs for *Daphnia* has only recently been investigated (E. Von Elert, *unpublished data*). Our results indicate that specific PUFAs (EPA and α-LA) are not mutually substitutable resources and that for interlake comparisons ratios of PUFAs might provide a good predictor of food quality under PUFA limitation. Seasonal succession between daphnids has been attributed to changing competitive outcome due to changes in food quality (Lynch 1978, DeMott 1983, Hu and Tessier 1995). DeMott (1998) presented data suggesting that PUFA-deficient diets could influence the outcome of competition between *Daphnia* species. Within the arena of PUFA lim-

itation, it remains to be tested if resource (PUFA) ratios hold considerable promise for improving our understanding of both ecosystem processes and population interactions in pelagic systems.

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