

A test of the role of polyunsaturated fatty acids in phytoplankton food quality for *Daphnia* using liposome supplementation

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Abstract

We conducted a series of experiments using the herbivorous zooplankter *Daphnia pulex* to investigate the nutritional importance of dietary polyunsaturated fatty acids (PUFAs). *Daphnia* were fed three different diets: (1) a PUFA-deficient cyanophyte mixture, (2) a cyanophyte mixture with fatty acid (FA) amendments, or (3) a PUFA-rich cryptophyte mixture. We devised a novel method using customized phosphatidylcholine liposomes to deliver specific FA amendments in a pure and bioavailable form that closely mimics their form in natural diets. We added FA-impregnated liposomes to the cyanophyte mixtures at levels equivalent to the observed differences in FA concentrations between the cyanophyte and cryptophyte mixtures. Liposome control amendments (without FAs added) had no effect on *Daphnia* growth. Eicosapentaenoic acid (EPA)-impregnated liposome amendments accounted for 30% of *Daphnia* somatic growth-rate differences and 38% of clutch-size differences between the cyanophyte and cryptophyte diets. Liposome supplementation of a FA mixture, which included a saturated fatty acid and the four ω 3 PUFAs most prevalent in cryptophytes but rare in cyanophytes, accounted for 59% of *Daphnia* somatic growth-rate differences and 47% of clutch-size differences between the cyanophyte and cryptophyte diets. Our study suggests that phytoplankton ω 3 PUFA, and especially EPA, content plays an important direct role in herbivorous zooplankton nutrition.

A considerable amount of research has focused on characterizing the importance of phytoplankton food quality for herbivorous zooplankton nutrition (Ahlgren et al. 1990; Gulati and DeMott 1997; Kilham et al. 1997). These and other studies demonstrate that herbivorous zooplankton growth rates are sometimes strongly correlated with the mineral and biochemical composition of the phytoplankton they consume (Müller-Navarra 1995; Sterner and Schulz 1998). The goal of much current research on this topic is to identify the specific mineral and biochemical constituents that actually cause these observed correlations. The results of zooplankton food-quality research have the potential to provide important insights for understanding trophic-level energy transfer in aquatic ecosystems. This in turn can lead to a better understanding of important processes such as food web interactions, eutrophication, and fisheries production (Brett and Goldman 1997; Brett and Müller-Navarra 1997).

Zooplankton food-quality studies are often approached from one of two perspectives. The first hypothesis emphasizes the role of elemental nitrogen and phosphorus in phytoplankton food quality (Sterner and Hessen 1994). The second hypothesis emphasizes the role of specific biochemicals such as amino acids, sterols, and polyunsaturated fatty acids (Brett and Müller-Navarra 1997; Klein Breteler et al. 1999). This study focuses on the second hypothesis and is designed to test the role that specific ω 3 polyunsaturated fatty acids (ω 3 PUFAs) play in determining phytoplankton food quality for herbivorous zooplankton. The PUFA limitation hypothesis is based on the observation that dietary ω 3 PUFA and especially eicosapentaenoic acid (EPA) or 20:5 ω 3 content is sometimes strongly correlated with *Daphnia* growth and egg production (Müller-Navarra 1995; Müller-Navarra et al. 2000; Park et al. 2002, 2003), and that phytoplankton taxa with high ω 3 PUFA content (e.g., diatoms and cryptophytes) are higher food quality than phytoplankton with a low ω 3 PUFA content (e.g., cyanophytes) (Ahlgren et al. 1990; Brett and Müller-Navarra 1997). Further evidence comes from aquaculture research showing that ω 3 PUFAs, especially EPA and docosahexaenoic acid (DHA) or 22:6 ω 3, are critical for maintaining high growth and reproductive rates, and high food conversion efficiencies within a wide variety of economically important marine and freshwater organisms (Levine and Sulkin 1984; Coutteau et al. 1997).

PUFAs that contain unsaturated bonds in the n3 or n6 position are synthesized by means of desaturases that are only found in plants (Vance and Vance 1985). Thus, ω 3 PUFAs are often referred to as essential fatty acids (EFAs) because they are considered to be essential components in

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animal diets. PUFAs have many important functions in cellular biology. PUFAs help regulate cell membrane fluidity and have also been linked with intracellular transport mechanisms, hormonal regulation, lipid metabolism, gene expression, and immune system functionality (Sargent et al. 1995). This study examines energetic coupling at the phytoplankton and herbivorous zooplankton interface because we believe that the transfer of ω 3 PUFAs at this level can have profound effects on energy transfer efficiency to upper levels in the food web (Brett and Müller-Navarra 1997).

To date, few researchers have conducted direct tests on the role of specific ω 3 PUFAs in determining phytoplankton food quality because it is difficult to deliver ω 3 PUFAs independent from phytoplankton cells. Several published studies on this topic have used complex lipid emulsion amendments to test the ω 3 PUFA limitation hypothesis (DeMott and Müller-Navarra 1997; Weers and Gulati 1997; Boersma et al. 2001; Park et al. 2003). Other studies have utilized carriers such as gum arabic-coated lipid microcapsules (Goulden et al. 1998) or bovine serum albumin (Von Elert and Wolffrom 2001) to deliver FA amendments. In an effort to build on prior research and place additional focus on the role of specific individual PUFAs, we devised a method using liposome carriers to deliver FA amendments in a pure and biologically available form that closely mimics the natural phytoplankton diets of herbivorous zooplankton.

Liposomes are spherical self-assembled colloidal particles that occur naturally and can be prepared artificially (Lichtenberg et al. 1988; Lasic 1993). Because liposomes resemble cell membranes in structure and composition, they were initially used as a tool to study biological membranes. Liposomes have been used extensively as chemical or pharmaceutical delivery agents (Bally et al. 1988; Gregoriadis 1993) and have been demonstrated to be useful for delivering ω 3 PUFAs in a variety of applications (Jenski et al. 1995; McEvoy et al. 1996). Furthermore, aquaculture research has shown that phosphatidylcholine (PC) liposomes are readily bioavailable to *Artemia nauplii* (Ozkizilcik and Chu 1994), and that they are therefore well suited to serve as carriers in experimental diets. For these reasons, we used PC liposome carriers to conduct a direct test of the importance of specific ω 3 PUFAs for herbivorous zooplankton food quality.

We determined the nutritional importance of individual FAs and FA mixtures by measuring *Daphnia* somatic growth rates and average individual clutch sizes at the primiparous instar in response to one of three diet types: (1) a PUFA-deficient cyanophyte mixture, (2) a cyanophyte mixture supplemented with specific fatty acids, or (3) a PUFA-rich cryptophyte mixture. Mixtures of cyanophytes and cryptophytes were used because they represent the lowest and highest food-quality phytoplankton groups for herbivorous zooplankton (Brett and Müller-Navarra 1997; Brett et al. 2000) and they are known to have very different PUFA content (Ahlgren et al. 1990; Brett and Müller-Navarra 1997). We used mixtures of phytoplankton monocultures (grouped as cyanophyte spp. and cryptophyte spp.) rather than single monocultures in order to better approximate the typically diverse phytoplankton assemblages encountered in natural systems.

Methods

All experiments were conducted using a clone of *Daphnia pulex* originally isolated from Clear Lake, California, and subsequently maintained in the lab for 5 yr prior to these experiments. *D. pulex* stock cultures were maintained on the green alga *Scenedesmus obliquus* in a growth chamber with a constant temperature of 18°C and a 14 h:10 h light:dark cycle.

Six phytoplankton monocultures, three cyanophytes (*Microcystis aeruginosa* 2063, *Microcystis aeruginosa* 2387, and *Synechococcus elongatus* obtained from the University of Texas culture bank) and three cryptophytes (*Cryptomonas ovata* 979/44, *Cryptomonas ovata* 979/61, and *Rhodomonas minuta* obtained from the University of Toronto Culture Collection) were maintained on L16 growth medium (Lindström 1983) supplemented with earth extract and B vitamins. These six phytoplankton monocultures were used to formulate cyanophyte spp. and cryptophyte spp. mixtures. Each phytoplankton mixture consisted of equal parts of the respective monocultures with a total concentration of 2.0 mg L⁻¹ phytoplankton dry weight. Phytoplankton biomass in the six monocultures was determined daily using calibrated fluorometry measurements and/or total suspended solids determinations.

Flow-through experiments were carried out in a 200-liter aquarium equipped with 12 partially submerged 120-ml chambers. A peristaltic pump was used to supply phytoplankton mixtures to the *Daphnia* in the chambers at a rate of 1.3 L d⁻¹ per chamber. *Daphnia* were fed one of the three diet types: (1) a PUFA-deficient cyanophyte mixture, (2) a cyanophyte mixture with liposome loaded with FA amendments, or (3) a PUFA-rich cryptophyte mixture. Food treatments were prepared fresh daily.

The liposomes (Sigma® sterile pyrogen-free preliposome formulation 5) were selected based on their purported temporal stability (at least 7 d), neutral charge, appropriate size (>1,000 nm in diameter; see Geller and Müller 1981), phase-change characteristics, and phosphatidylcholine distearoyl composition (C₄₄H₈₈NO₈P). Liposomes were formulated and loaded by adding 1.0 ml of sterile deionized water heated to 62°C to the condensed phosphatidylcholine compound. Solvents used in the storage of free-form FAs (Sigma®) were evaporated under a stream of nitrogen gas and FAs were then recombined in a standardized solvent mixture (2 μ l of methanol per 1.0 mg FA) to facilitate encapsulation. The 1.0-ml hydrated liposome solution was then added to the FA solution and encapsulation was accomplished by vortexing the mixture at moderate speed and room temperature for 15 min.

In general, liposome FA treatments consisted of individual or combined FA amendments designed to mimic the individual or combined differences in the FA content of the cyanophyte and cryptophyte spp. mixtures. For example, if the cyanophyte mixture contained 0.5 μ g EPA mg C⁻¹ and the cryptophyte mixture contained 15.0 μ g EPA mg C⁻¹, liposomes impregnated with EPA were added to the cyanophyte mixture at a concentration of 14.5 μ g EPA mg C⁻¹.

Twelve hours prior to the start of each experiment, egg-

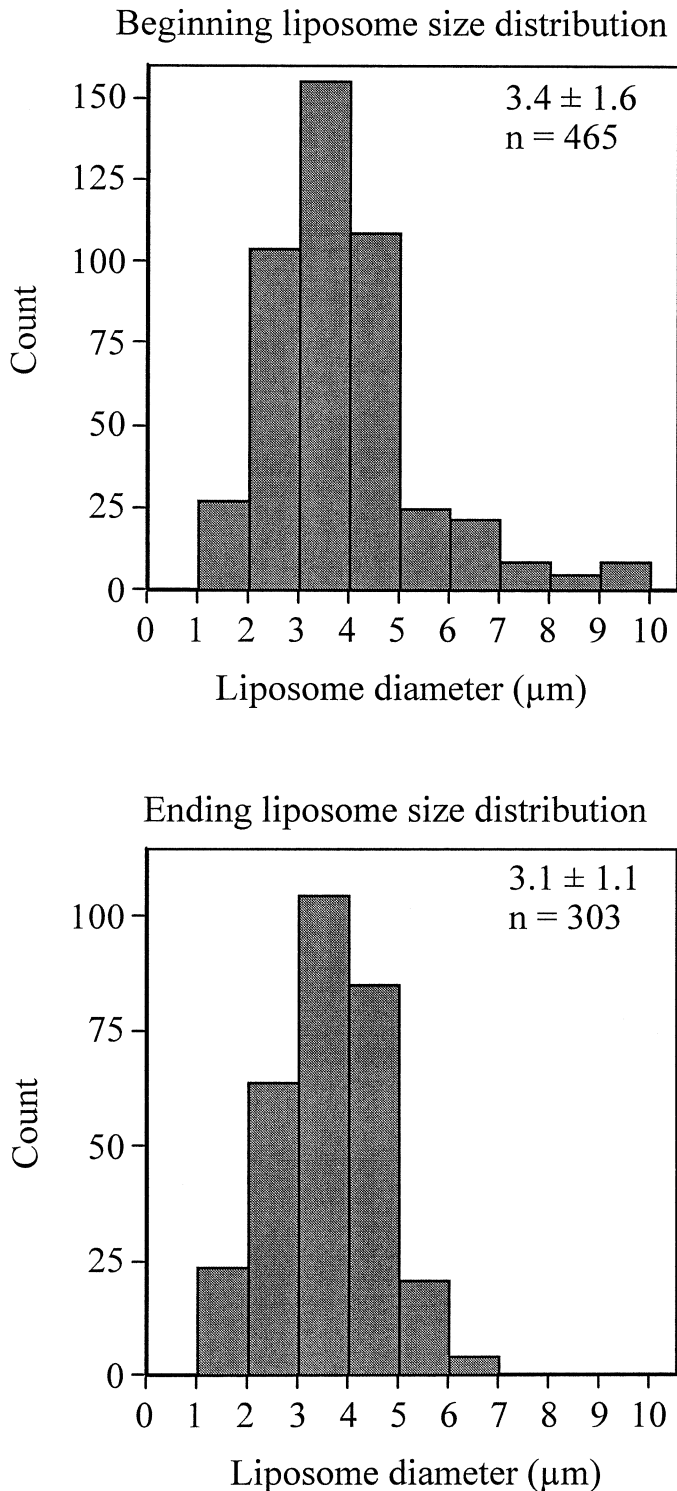


Fig. 1. Size distribution of liposome subsamples taken on day 1 of experiments (top graph, pooled from three experiments) and on the last day (day 6) of experiments (bottom graph, pooled from two of the same three experiments). The liposomes had an average diameter of $3.4 \pm 1.6 \mu\text{m}$ on day 1 of the experiments and $3.1 \pm 1.1 \mu\text{m}$ on day 6 of the experiments. The between-experiment differences in liposome size distribution were negligible.

bearing *Daphnia* were separated from the stock culture and placed into individual 20-ml scintillation vials with *S. obliquus* as food. At the beginning of each experiment, neonates from this ~ 6 -h-old cohort were randomly selected and transferred to each of the 12 experimental chambers. A subsample of approximately 15–20 neonates was simultaneously dried and weighed on a Cahn Microbalance (Model C33) to provide an initial neonate biomass estimate. Eight neonates were placed in each chamber, and the chambers were provided a constant flow for each of the specific dietary treatments. Each experiment lasted 6 d. At the conclusion of each experiment, *Daphnia* were collected, measured individually for length and clutch size (eggs per individual) under a microscope and then each daphnid was dried (24 h at 105°C) and weighed (in groups of four individuals from each replicate) to obtain the average individual weight per replicate. Somatic growth rates (g) were calculated accordingly as $g = \ln(W_f/W_i)/t$, where W_i is the initial animal weight, W_f is the final animal weight and t is the duration of each experiment in days. Mortality was almost always less than 13% in these replicates.

A subsample of the liposome preparations was collected during three of the five experiments to determine the liposome size distribution. On two of these occasions, a subsample was collected at the end of the experiments to investigate temporal changes in liposome size distribution during the course of the experiments. The pooled measurements of pre- and postexperiment liposome size distributions are presented in Fig. 1. When first prepared (day 1 of each experiment) the liposomes had an average size of 3.4 ± 1.6 (± 1 standard deviation [SD]) μm in diameter, $n = 465$, whereas at the end of the 6-d experiments, these preparations had an average size of $3.1 \pm 1.1 \mu\text{m}$ in diameter, $n = 303$. The only practical difference between the liposome size distributions on days 1 and 6 of these experiments is that the liposomes greater than $6 \mu\text{m}$ in diameter were unstable over multiday periods. Samples from the phytoplankton food treatments were collected on day 5 of each experiment and prepared for biochemical analyses. Particulate matter was collected in duplicate on precombusted glass fiber filters (Whatman GF/C). Chlorophyll *a* was determined using a Turner 10-AU fluorometer with acid correction for phaeophytin (Marker et al. 1980). Fatty acid methyl esters were analyzed with a gas chromatograph (HP6890) equipped with a programmable temperature vaporizer injector (PTV), a fused silica capillary column (DB-WAX, J&W Scientific; $30 \text{ m} \times 0.32 \text{ mm}$ with $0.25\text{-}\mu\text{m}$ film thickness), and a flame ionization detector (Kattner and Fricke 1986). Five microliters of sample were injected, with helium used as the carrier gas. The temperature program applied was as follows: 40°C held for 5 min, then heated up at $10^\circ\text{C min}^{-1}$ to 150°C , held for 5 min, then heated up at 1°C min^{-1} to 220°C , where it was kept for 20 min. Individual fatty acid identifications were based on the retention times of fatty acid methyl ester standards (Sigma, Supelco, Alltech) dissolved in n-hexane. Quantification was performed with an internal standard (21:0) and quantitative mixes (Alltech) to calculate response factors for each fatty acid analyzed.

We conducted five sets of experiments to test the PUFA

limitation hypothesis. Each experiment type was run twice, with the exception of experiment set 1, which was run once.

Experiment 1: Cyanophyte mixture versus cryptophyte mixture—The first experiment measured the relative food quality of two phytoplankton mixtures (a PUFA-deficient cyanophyte mixture and a PUFA-rich cryptophyte mixture) delivered at six different proportions. This experiment included six treatments that ranged in increments of 20% from 100/0% to 0/100% cyanophytes to cryptophytes. Each treatment consisted of two replicates. The concentration of all combinations was held constant at 2.0 mg L⁻¹ phytoplankton dry weight.

Experiment set 2: Cyanophytes + PC versus cyanophytes + PC + EPA—This experiment was designed to test the impact of PC liposome amendments (without FAs loaded) and amendments of PC liposomes loaded with EPA on *Daphnia* somatic growth rates and egg production. This experiment employed four treatments with three replicates per treatment. The four treatments were (1) cyanophyte mixture without liposomes added, (2) cyanophyte mixture with PC liposomes alone added (cyanophytes + PC), (3) cyanophyte mixture with liposomes impregnated with EPA added (cyanophytes + PC + 20:5 ω 3), and (4) cryptophyte mixture without liposomes added.

Experiment set 3: Cyanophytes + PC + palmitic acid versus cyanophytes + PC + EPA—The purpose of this experiment was to compare *Daphnia* responses to amendments of a saturated fatty acid (palmitic acid or 16:0) and a highly unsaturated fatty acid (EPA). In this experiment, three treatments (i.e., cyanophytes, cyanophytes + PC + 20:5 ω 3 and cryptophytes) were rerun from experiment 2. In addition to these treatments, a saturated fatty-acid treatment (cyanophytes + PC + 16:0) was added instead of the liposome control that was used in experiment 2. Each treatment was carried out in triplicate.

Experiment set 4: Cyanophytes + PC + individual PUFAs versus cyanophytes + PC + PUFA mixture—This experiment determined the nutritional value of three single ω 3 PUFA amendments (18:3 ω 3, 20:5 ω 3, 22:6 ω 3) and a mixture of the same three ω 3 PUFAs to test for additive or synergistic effects. The six treatments for this experiment were (1) cyanophyte mixture without liposomes added, (2) cyanophyte mixture with liposomes impregnated with 18:3 ω 3 added (cyanophytes + PC + 18:3 ω 3), (3) cyanophyte mixture with liposomes impregnated with DHA added (cyanophytes + PC + 22:6 ω 3), (4) cyanophyte mixture with liposomes impregnated with EPA added (cyanophytes + PC + 20:5 ω 3), (5) cyanophyte mixture with liposomes impregnated with 18:3 ω 3, 20:5 ω 3, and 22:6 ω 3 added (cyanophytes + PC + 18:3 ω 3 + 20:5 ω 3 + 22:6 ω 3), and (6) cryptophyte mixture without liposomes. Each treatment was run in duplicate.

Experiment set 5: Addition of a mixture that approximates total FA content differences—This experiment was designed to test the nutritional importance of the five most abundant

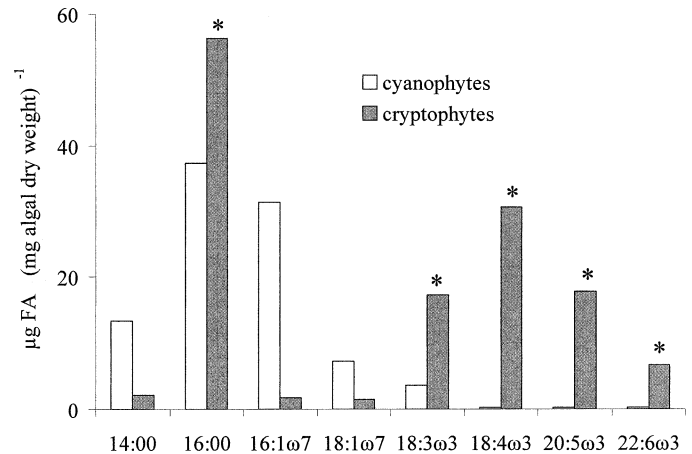


Fig. 2. Fatty acids profiles for the cyanophyte and cryptophyte treatments. The fatty acids plotted here represent major constituents (>10% of the total fatty acid concentrations) of either the cyanophyte spp. or cryptophyte spp. mixtures. The FAs added as amendments to the cyanophyte mixture are marked with asterisks. Note the cyanophytes are deficient in ω 3 PUFAs relative to the cryptophytes.

FAs (16:0, 18:3 ω 3, 18:4 ω 3, 20:5 ω 3, and 22:6 ω 3) in the cryptophyte treatment. These five fatty acids accounted for approximately 65% of the total cryptophyte FA content, and they were also relatively rare in the cyanophyte mixture. The three treatments in this experiment were (1) cyanophyte mixture without liposomes added, (2) cyanophyte mixture with liposomes impregnated with five individual fatty acids added (cyanophytes + PC + 16:0 + 18:3 ω 3 + 18:4 ω 3 + 20:5 ω 3 + 22:6 ω 3), and (3) cryptophyte mixture without liposomes. Four replicates per treatment were used in this experiment.

Statistical analyses—*Daphnid* growth rates and clutch sizes were statistically analyzed in each set of experiments using two-factor analysis of variance (ANOVA). Statistical significance between treatments was assessed using Dunnett's *t*-tests ($P < 0.01$).

Results

Phytoplankton fatty acid composition—A select group of the FAs that were measured in the cyanophyte and cryptophyte mixtures is depicted in Fig. 2. We focused on this group of five fatty acids, measured during experiment 1, because they are the fatty acids that were the most abundant in the cryptophyte mixture and were relatively scarce in the cyanophyte mixture. The FA concentration differences of the cyanophyte and cryptophyte mixtures in this experiment served as the target FA concentrations to be amended (measured as the difference in FA concentration between the cyanophyte mixture and the cryptophyte mixture) in the supplementation experiments. In general, we were able to come within 9% of all target FA concentration differences and within 16% of all actual FA concentration differences for each liposome FA supplementation experiment. Liposome amendments of the target fatty acids did not have noticeable

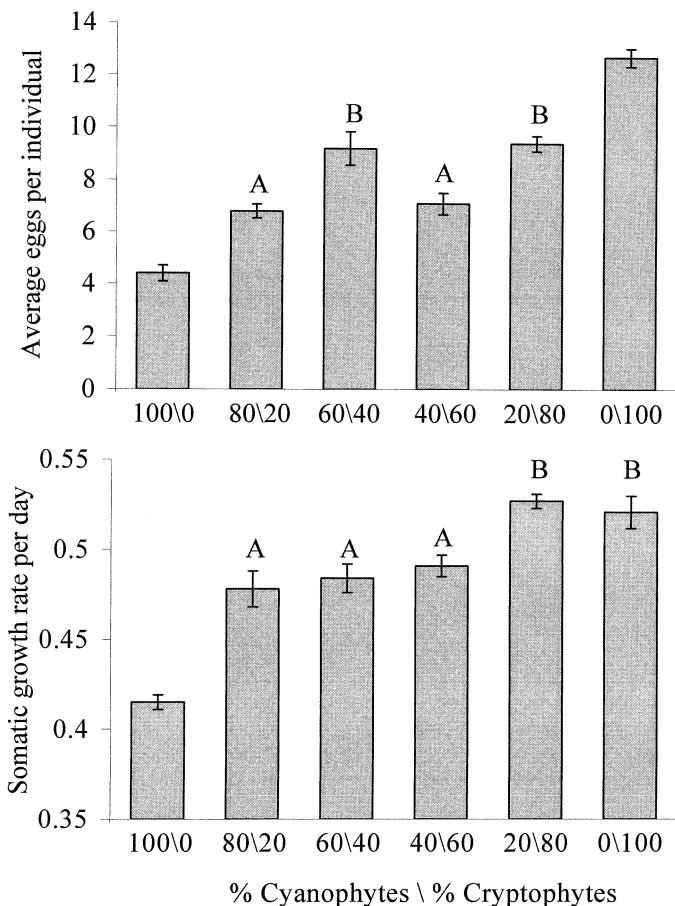


Fig. 3. Results from experiment 1, which were obtained by varying the ratio of cyanophytes to cryptophytes in the daphnid diets. The total concentration of the combined phytoplankton mixtures was kept at 2.0 mg L^{-1} phytoplankton dry weight. Similar superscripts indicate no statistical difference was detected between treatments (Dunnett's t -test, $P < 0.01$; Table 1).

effects on the concentrations of other fatty acids in the food treatments.

Experiment 1: Cyanophyte mixture versus cryptophyte mixture—The first addition of cryptophytes to the cyanophyte mixture (80:20% cyanophytes: cryptophytes) increased individual clutch sizes and somatic growth rates compared with the 100% cyanophyte mixture (Fig. 3). The somatic growth rates observed for the 80/20%, 60/40% and 40/60% treatments were higher than for the 100% cyanophyte treatment but not significantly different from each other. Finally, the somatic growth rates measured for the 20/80% and 0/100% treatments were similar to each other, but significantly higher than the other four treatments. The mean clutch size had a similar, though not identical, trend as somatic growth.

Particulate phosphorus levels in the phytoplankton food treatments—The average C:P molar ratio in the cyanophyte mixture treatments was 127 ± 8 (± 1 SD, $n = 16$). Amendments of PC liposomes to cyanophyte mixtures (with and

without fatty acids) decreased the average C:P ratio slightly to 111 ± 9 ($n = 34$). The average C:P ratio in the cryptophyte treatments was 152 ± 18 ($n = 16$).

Experiment set 2: Cyanophytes + PC versus cyanophytes + PC + EPA—*Daphnia* average clutch size and somatic growth rate did not change in the PC liposome control treatment relative to the cyanophyte mixture treatment (Fig. 4). However, average clutch size and somatic growth rate were significantly enhanced in the cyanophytes + PC + EPA and cryptophyte mixture treatments relative to the cyanophyte mixture and liposome control treatments. *Daphnia* fed the cyanophyte mixture amended with EPA-impregnated PC liposomes had larger primiparous clutches and grew faster than *Daphnia* fed only cyanophytes. The cyanophytes + PC + EPA treatment accounted for approximately 30% of the somatic growth-rate and 37% of the mean clutch-size differences between the cyanophyte and cryptophyte treatments. In general, nearly identical between-treatment differences were noted for the clutch-size and somatic growth-rate parameters. The results of a two-factor ANOVA showed experimental effects were negligible and treatment effects were very strong.

Experiment set 3: Cyanophytes + PC + palmitic acid versus cyanophytes + PC + EPA—The results for this experiment (Fig. 5) show the cyanophytes + PC + palmitic acid treatment had a significant but small (17% increase) effect on *Daphnia* growth rates and a nonsignificant effect on average clutch size relative to the cyanophyte mixture. The cyanophytes + PC + EPA treatment was found to be significantly different from both the cyanophyte mixture and the cyanophytes + PC + palmitic acid treatments for both response parameters. The cyanophytes + PC + EPA treatment accounted for 30% of the somatic growth-rate and 41% of the mean clutch-size differences between the cyanophyte and cryptophyte treatments. The between-treatment differences for growth rate and average clutch size were significantly different. The results of the two-factor ANOVA show that experimental effects were small and treatment effects were very strong.

Experiment set 4: Cyanophytes + PC + individual PUFAs versus cyanophytes + PC + PUFA mixture—Results from experiment 4 (Fig. 6) show that the cyanophytes + PC + 18:3 ω 3 treatment accounted for 14% of the somatic growth-rate differences between the cyanophyte and cryptophyte treatments; however, it did not have a significant effect on mean clutch size. The cyanophytes + PC + 22:6 ω 3 treatment accounted for approximately 13% of the somatic growth-rate and 11% of the mean clutch-size differences between the cyanophyte and cryptophyte treatments. Both the cyanophytes + PC + 20:5 ω 3 treatment and the cyanophytes + 18:3 ω 3 + 20:5 ω 3 + 22:6 ω 3 treatment had significant effects on average clutch size and somatic growth rate compared with the cyanophyte treatment. These treatments accounted for 36 and 37% of the mean clutch-size differences, respectively, and 31 and 30% of the somatic growth-rate differences, respectively. The between-treatment differences for the clutch-size and growth-rate parameters

Table 1. Two-factor ANOVA results for daphnid clutch-size and somatic growth-rate response to the fatty acid supplementation treatments. Experiment denotes the statistical differences between duplicate experiments within an experiment type. df, degrees of freedom; SS, sums of squares; Percent SS, variance explained.

	Source	df	SS	F-test	P value	Percent SS
Clutch size						
Experiment 1	Between Trt.	5	43.6	28.30	0.0004	96.0
	Within Trt.	6	1.84			4.0
Experiment 2	Experiment	1	6.56	30.70	0.0001	2.9
	Treatment	3	211	329.00	0.0000	94.4
	Interaction	3	2.46	3.84	0.0304	1.1
	Error	16	3.43			1.5
Experiment 3	Experiment	1	1.07	4.15	0.0585	0.4
	Treatment	3	291	376.00	0.0000	96.3
	Interaction	3	5.84	7.54	0.0023	1.9
	Error	16	4.13			1.4
Experiment 4	Experiment	1	0.392	0.78	0.3967	0.2
	Treatment	4	233	116.00	0.0000	97.7
	Interaction	4	0.093	0.05	0.9952	0.0
	Error	10	5			2.1
Experiment 5	Experiment	1	22.1	4.81	0.0418	7.6
	Treatment	2	180	19.60	0.0001	62.2
	Interaction	2	4.56	0.50	0.6174	1.6
	Error	18	82.8			28.6
Somatic growth rate						
Experiment 1	Between Trt.	5	0.0110	14.30	0.0028	91.7
	Within Trt.	6	0.00100			8.3
Experiment 2	Experiment	1	0.00000	3.39	0.0844	0.0
	Treatment	3	0.147	908.00	0.0000	98.0
	Interaction	3	0.00200	11.70	0.0003	1.3
	Error	16	0.00100			0.7
Experiment 3	Experiment	1	0.00000	2.86	0.1101	0.0
	Treatment	3	0.161	904.00	0.0000	99.4
	Interaction	3	0.00000	0.60	0.6269	0.0
	Error	16	0.00100			0.6
Experiment 4	Experiment	1	0.00001	0.07	0.7998	0.0
	Treatment	4	0.0938	124.30	0.0000	98.0
	Interaction	4	0.00001	0.01	0.9997	0.0
	Error	10	0.00189			2.0
Experiment 5	Experiment	1	0.00003	0.05	0.8344	0.0
	Treatment	2	0.194	155.00	0.0000	94.4
	Interaction	2	0.00024	0.19	0.8303	0.1
	Error	18	0.0113			5.5

were fairly consistent. The results of a two-factor ANOVA showed that the experimental effects were small and the treatment effects were very strong.

Experiment 5: Addition of a mixture that approximates total FA content differences—The results of this set of experiments (Fig. 7) show that *Daphnia* average clutch size and growth rate for the cyanophytes + PC + FAs treatment was significantly higher than the cyanophyte treatment; however, reproduction and somatic growth rate in the cryptophyte treatment was still significantly higher than for the cyanophytes + PC + FAs treatment. The cyanophytes + PC + FAs treatment accounted for approximately 59% of the somatic growth rate and 47% of the mean clutch-size difference between the cyanophyte and cryptophyte treatments. The between-treatment differences for the two experiments were fairly consistent, although *Daphnia* tended to have

larger clutches in the second experiment. The results of a two-factor ANOVA showed that the experimental effects were moderate and the treatment effects were strong.

Discussion

These experiments showed that adding liposomes supplemented with EPA to a cyanophyte mixture significantly increased *Daphnia* growth and egg production. These results strongly support the hypothesis that this ω 3 PUFA plays an important role in determining phytoplankton food quality for *Daphnia* (Ahlgren et al. 1990; Müller-Navarra 1995; Brett and Müller-Navarra 1997; Müller-Navarra et al. 2000). Amendments of EPA alone accounted for approximately 30% of the somatic growth-rate and 38% of the egg production differences, on average, between the cyanophyte and cryptophyte treatments. Furthermore, 59% of the somatic

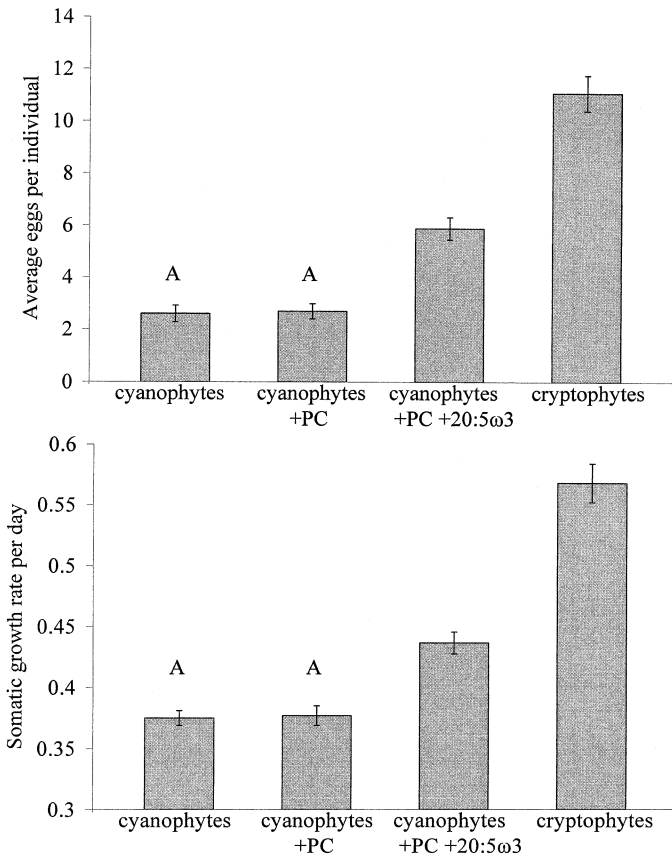


Fig. 4. Results from experiment 2 involving amendments of empty liposomes (control) and liposomes loaded with EPA added to the cyanophytes treatment. All treatments were kept at a concentration of 2.0 mg L^{-1} phytoplankton dry weight. Similar superscripts indicate no statistical difference was detected between treatments (Dunnett's *t*-test, $P < 0.01$; Table 1).

growth-rate and 47% of the egg production differences between these treatments were accounted for by a FA amendment that included palmitic acid and the four ω_3 PUFAs that were the most prevalent in the cryptophytes.

Interestingly, additions of α -LA or DHA produced only small growth and reproduction responses in *Daphnia pulex* compared with the EPA response. This suggests that neither α -LA nor DHA are completely adequate substitutes for EPA. In addition, a mixture of α -LA, DHA, and EPA did not produce additive effects beyond the benefits conferred by EPA alone (Experiment 4, Fig. 6). This suggests these FAs may be involved in the same metabolic pathways for *Daphnia*, though it may also be that it is the resource ratios of these PUFAs and not necessarily their absolute quantities that determine growth limitation (Wacker and Von Elert 2001). These results are consistent with the observations of Müller-Navarra (1995) and Müller-Navarra et al. (2000), in which it was found that EPA was the single fatty acid in seston that correlated most strongly with *Daphnia* growth rates. This result also suggests daphnids have low essential fatty acid conversion capacities. In contrast, when stearidonic acid ($18:4\omega_3$) was added to the FA mixture in experiment 5 (Fig. 7), significant additive effects were observed. This suggests

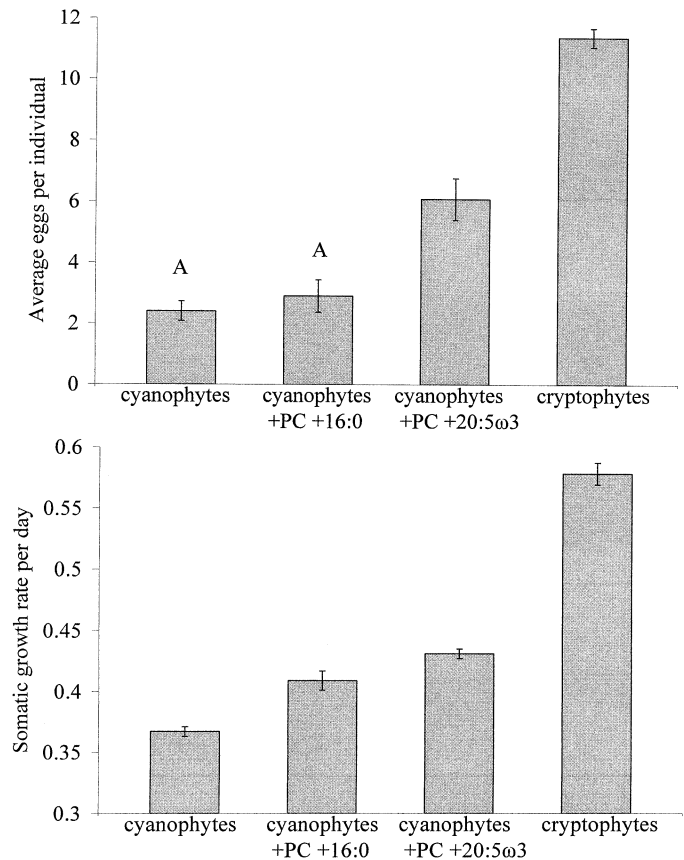


Fig. 5. Results from experiment 3 involving amendments of liposomes loaded with palmitic acid and EPA to the cyanophyte mixture. All treatments were kept at a concentration of 2.0 mg L^{-1} phytoplankton dry weight. Similar superscripts indicate no statistical difference was detected between treatments (Dunnett's *t*-test, $P < 0.01$; Table 1).

that, in contrast with α -LA, stearidonic acid may be a more direct precursor for EPA in *Daphnia pulex*.

However, we cannot rule out the possibility that the addition of palmitic acid ($16:0$) to the FA-mixture in experiment 5 accounted for some of *Daphnia's* growth response. Individual amendments of palmitic acid in experiment 3 accounted for approximately 17% of the difference in daphnid somatic growth between the cyanophyte and cryptophyte treatments; however, the palmitic acid amendment in experiment 3 did not have an effect on *Daphnia* reproduction. Nevertheless, the modest growth benefit produced by the palmitic acid amendment was somewhat surprising because this saturated fatty acid was not expected to be a limiting factor in *Daphnia* metabolism. However, fatty acid synthesis rates are very low in daphnids (Goulden and Place 1990), and our observation suggests that *Daphnia pulex* has not only a requirement for a specific unsaturated fatty acid profile in position *sn*-2 but also for saturated fatty acids inserted in the *sn*-1 position of the phospholipids. As the pure liposomes only consisted of stearic acid ($18:0$), addition of $16:0$ may foster membrane synthesis.

The outcome of the supplementation experiments is consistent with the results from the phytoplankton mixture ex-

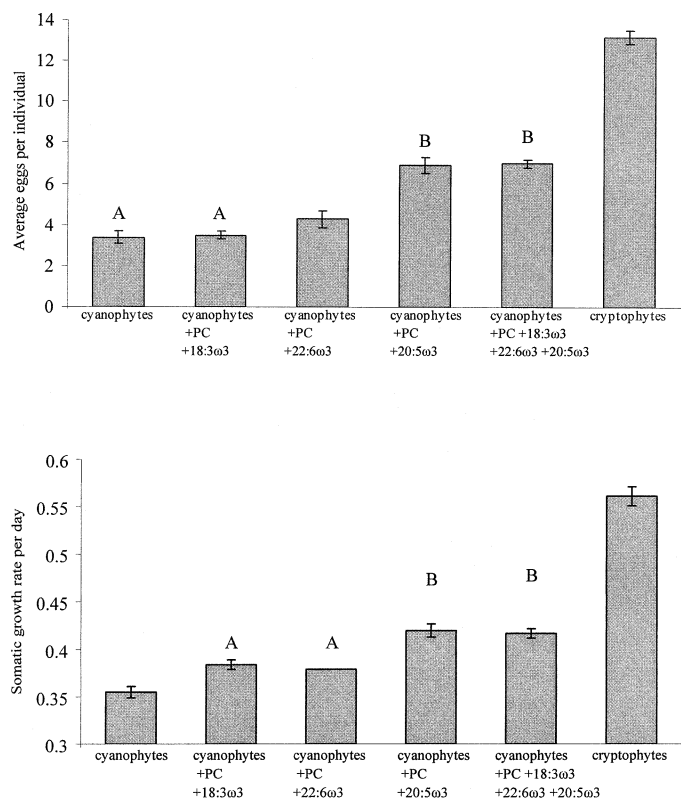


Fig. 6. Results from experiment 4 comparing three individual ω 3 PUFA supplements with a mixture of those ω 3 PUFAs (EFA mixture). All treatments were kept at a concentration of 2.0 mg L^{-1} phytoplankton dry weight. Similar superscripts indicate no statistical difference was detected between treatments (Dunnett's *t*-test, $P < 0.01$; Table 1).

periment 1, which exhibit a stair-step increase in growth and egg production with increasing availability of cryptophytes. When the cryptophyte mixture was combined with the cyanophyte mixture at a ratio of 20%, the growth parameters increased significantly up to a level that was only enhanced by cryptophyte additions of 60% (growth rate) or 80% (clutch size). Because the cryptophytes have a very high ω 3-PUFA, and especially EPA, content adding 20% cryptophytes to the cyanophyte mixture increased the EPA level in the food mixture from below detection to $3 \mu\text{g EPA mg C}^{-1}$. This level is already high and perhaps saturating compared with *Daphnia* growth and egg production responses to seston EPA content (Müller-Navarra et al. 2000). This first step is most likely due to essential fatty acids, such as EPA, as the comparison with experiment set 5 clearly suggests. The second significant increase in this stair-step function may be due to a different limiting substance with a different threshold level for dietary availability (i.e., more than one type of essential FA or another substance is required for maximal growth), and cryptophytes seem to contain both in optimal concentrations. The nature of this potentially limiting substance needs further investigation.

One observation that can be made when comparing the results of this study with results from other PUFA-supplementation studies is that studies that have used lipid emul-

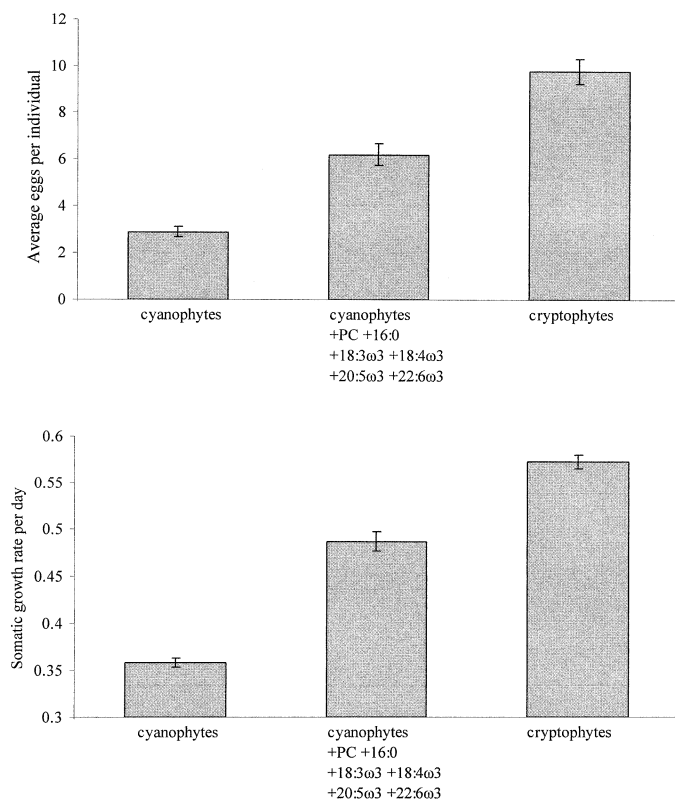


Fig. 7. Results from experiment 5, involving the amendment of a multi-FA mixture (FA-mixture consisting of palmitic acid, α -LA, stearidonic, EPA, and DHA) to the cyanophytes mixture. All treatments were kept at a concentration of 2.0 mg L^{-1} phytoplankton dry weight.

sions containing highly unsaturated fatty acids (DeMott and Müller-Navarra 1997; Weers and Gulati 1997; Boersma et al. 2001; Park et al. 2003) have generally concluded that the addition of PUFAs to the daphnid diet has a stimulatory effect on growth, whereas prior to this series of experiments, studies that have used artificial carriers to deliver FA supplements have concluded that PUFAs do not have a stimulatory effect on daphnid growth (Goulden et al. 1998; Von Elert and Wolffrom 2001). The study by Goulden et al. (1998) used gum arabic-coated lipid microcapsules to deliver 18:2 ω 6, 18:3 ω 3, 16:0, and 18:1 supplements to natural lake seston. They did not include 20:5 ω 3, the fatty acid for which we observed the largest response, and this alone may have resulted in an important difference between our studies. The study by Von Elert and Wolffrom (2001) used bovine serum albumin (BSA) beads to deliver 18:3 ω 3, 18:4 ω 3, 20:4 ω 6, and 20:5 ω 3, and they concluded that supplementing *Synechococcus elongatus* with PUFAs did not improve *Daphnia* growth. The most important distinction between the methods employed by Von Elert and Wolffrom (2001) and our study is that Von Elert and Wolffrom delivered specific fatty acids to *Daphnia* using BSA and we delivered fatty acids using liposomes. Another notable difference between our experimental design and theirs is that we used a mixture of three cyanophytes whereas Von Elert and Wolffrom used a single cyanophyte monoculture as a baseline food treat-

ment. It should also be noted that the *Daphnia pulex* clone used in our experiments originated from a lake that is usually dominated by cyanobacteria. Taken together, these factors may help to explain why *Daphnia pulex* in our study grew and reproduced much more successfully on a cyanophyte diet than did *Daphnia galeata* in their study.

We do not have any direct evidence to explain why PUFAs delivered via phosphatidylcholine liposomes would stimulate daphnid growth more so than PUFAs delivered via other carriers; thus, this topic deserves further research. It is possible the liposomes used in our study may improve the digestibility of PUFAs in the daphnid gut (Ozkizilcik and Chu 1994) and/or provide oxidative stability to the PUFA supplements (McEvoy et al. 1995; Nara et al. 1998; Reini-kainen et al. 2001). Aquaculture studies have shown that the utility and bioavailability of dietary lipid amendments can be strongly affected by not only the type of lipid but also by the specific form or medium in which the lipid is delivered in the diet (Coutteau and Sorgeloos 1997). Because metabolic pathways often involve specific forms of the input chemical in order to trigger a full response, it can be reasoned that FAs are more bioavailable in the gastrointestinal tract of daphnids when they are incorporated within membrane lipids such as phosphatidylcholine. For example, it has been shown that the eicosanoid class of hormones are produced from PUFAs through enzymatic procedures that specifically target membrane-incorporated PUFAs (Diez et al. 1994).

In summary, this study clearly illustrates beneficial effects of artificial dietary ω 3 PUFA supplementation on *Daphnia* growth and reproduction. This is a valuable step forward in the discussion about correlation versus causation and the effects of ω 3 PUFAs on zooplankton growth, reproduction, and energy transfer efficiency. Our results also highlight the importance of the form in which fatty acids are supplemented in the artificial diets. This study strongly supports the hypothesis that PUFAs play an important role in determining phytoplankton food quality for herbivorous zooplankton.

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