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Nutritional constraints at the cyanobacteria–*Daphnia magna* interface: The role of sterols

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Abstract

In past decades, a considerable amount of research has been conducted to elucidate the factors that affect the carbon transfer across the cyanobacteria–*Daphnia* interface. It is well accepted that cyanobacteria are a nutritionally inadequate food source for cladocerans, but the underlying mechanisms are still controversial. Morphological properties, toxicity, and the absence of essential lipids, i.e., polyunsaturated fatty acids (PUFAs) and sterols, are discussed as the most important factors that account for this nutritional inadequacy. Here, we conducted standardized growth experiments with the herbivore *Daphnia magna* feeding on coccal, filamentous, and putatively toxic cyanobacterial strains comprising the genera *Synechococcus*, *Anabaena*, *Aphanizomenon*, and *Microcystis* and on a cyanobacterial mixture containing strains of these genera. The relative importance of PUFAs and sterols in determining the food quality of cyanobacteria for *Daphnia* was assessed by supplementation of eicosapentaenoic acid- and/or cholesterol-containing liposomes to the cyanobacterial carbon. We provide evidence that somatic growth of daphnids on coccal as well as on filamentous cyanobacteria is primarily constrained by the absence of sterols, provided that the cyanobacterial carbon is readily ingested and nontoxic. The absence of PUFAs in cyanobacteria appears to be of minor importance for somatic growth but potentially affects egg production in *Daphnia*. Thus, the absence of sterols has to be considered a major food-quality constraint that potentially affects the efficiency of carbon transfer across the cyanobacteria–*Daphnia* interface.

Trophic interactions at the autotroph–herbivore interface significantly affect the efficiency of carbon transfer to higher trophic levels in freshwater ecosystems. Cladocerans of the genus *Daphnia* are the dominant herbivores in lakes and ponds; owing to their abundance and their high grazing activity on the phytoplankton they provide a crucial link between primary and secondary production. However, the carbon transfer efficiency at the phytoplankton–*Daphnia* interface is often constrained by the predominance of nutritionally inadequate food sources.

Cyanobacteria are well known for their nutritional inadequacy for freshwater cladocerans (De Bernardi and Giussani 1990; Wilson et al. 2006). In particular in eutrophic lakes, where cyanobacteria often dominate the phytoplankton, carbon is transferred inefficiently to crustacean grazers. As a result, an accumulation of cyanobacterial biomass occurs (cyanobacterial bloom), which is often associated with hazards to human health and livestock and reduced recreational quality of water bodies (Carmichael 1994).

In past decades, reasons determining the low assimilation of cyanobacterial carbon by *Daphnia* have been extensively studied, and three major food-quality constraints have been revealed: (1) grazing resistance, (2) toxicity, and (3) a deficiency in essential nutrients. Grazing resistance due to morphological properties, such as the formation of large filaments or cell colonies, is regarded as the most important food-quality constraint. Mechanical interference with the filtering process of daphnids not only hampers ingestion, but also is associated with higher rejection and respiration rates and, therewith, increased energetic costs (Porter and McDonough 1984). Large *Daphnia* species (e.g., *Daphnia magna*) have been shown to be more sensitive to filamentous cyanobacteria than smaller sized *Daphnia* species because of their larger carapace gap, i.e., smaller sized *Daphnia* have a greater ability to avoid the filtration of large filaments. This might also explain the often observed shift from large- to small-bodied cladocerans during cyanobacterial bloom conditions (Porter and McDonough 1984; DeMott et al. 2001).

Toxin production has been reported in a large number of cyanobacterial species. Toxin content, however, varies significantly among strains and even between clones of the same isolate (Dow and Swoboda 2000). Conventional-

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ly, cyanobacterial toxicity is assessed by mouse bioassay but does not necessarily reflect toxicity to zooplankton grazers. However, cyanobacterial toxins have been shown to harm *Daphnia*, the most prominent example being the microcystins produced by several strains of the genus *Microcystis* (DeMott et al. 1991; Rohrlack et al. 2001).

Recently, food-quality research has focused on the lipid requirements of the major zooplankton taxa. In arthropods, long-chain polyunsaturated fatty acids (PUFAs) and sterols are essential dietary compounds that cannot be synthesized de novo (Harrison 1990; Grieneisen 1994); both are integral parts of cell membranes and both serve as precursors of many bioactive molecules. The long-chain PUFAs arachidonic acid (ARA) and eicosapentaenoic acid (EPA), for instance, are precursors of prostaglandins, which are relevant in arthropod reproduction (Harrison 1990). Sterols, on the other hand, are precursors of steroid hormones, such as ecdysteroids, which are involved in the process of molting (Grieneisen 1994). A correlative field study has suggested that the absence of long-chain PUFAs, in particular EPA, is responsible for the poor assimilation of cyanobacterial carbon by *Daphnia* (Müller-Navarra et al. 2000). In this study, however, the strong predictive power of the availability of EPA for *Daphnia* growth may not necessarily reflect a causal dietary deficiency. Cyanobacteria, as prokaryotes, differ from eukaryotic algae not only in the absence of EPA (Gugger et al. 2002), but also in the absence of sterols (Volkman 2003, 2005; Summons et al. 2006). Hence, in seston dominated by cyanobacteria, low concentrations of EPA and of sterols will be highly correlated, which renders a sterol limitation possible. In another field study, De Lange and Arts (1999) correlated biochemical variables of natural seston with *Daphnia* growth rates. They found a significant positive correlation of the growth of *Daphnia* with the sterol content of natural seston.

The correlative field data of Müller-Navarra et al. (2000) were corroborated experimentally by Ravet et al. (2003), who reported that the growth of *Daphnia pulex* on a cyanobacterial diet is improved by EPA supplementation. In contrast, however, by assessing PUFAs and sterols it was demonstrated that the growth of daphnids on a cyanobacterial diet is primarily constrained by the absence of sterols and that PUFAs become limiting only when the shortage of sterols has been overcome by sterol supplementation (von Elert et al. 2003).

Here, in order to shed light onto conflicting data regarding the mechanisms of food-quality constraints, we conducted standardized growth experiments with the herbivore *D. magna* feeding on different cyanobacterial strains of the genera *Synechococcus*, *Anabaena*, *Aphanizomenon*, and *Microcystis* and on a cyanobacterial mixture containing strains of these genera. The relative importance of PUFAs and sterols in determining the food quality of cyanobacteria for *Daphnia* was investigated by supplementation of EPA- and/or cholesterol-containing liposomes to the cyanobacterial carbon. The obtained results are integrated into the more classical theories of food-quality constraints caused by morphological properties or toxicity.

Material and methods

Cultivation of the food organisms—The green alga *Scenedesmus obliquus* (SAG 276-3a) was used as food for the stock cultures of the daphnids. It was grown in batch cultures in Cyano medium (Jüttner et al. 1983) and harvested in the late-exponential growth phase. For the growth experiments, *Synechococcus elongatus* (SAG 89.79), *Anabaena variabilis* (ATCC 29413), *Anabaena cylindrica* (SAG 1403-2), *Aphanizomenon flos-aquae* (CCAP 1401-1 and UTEX LB 2384), *Microcystis aeruginosa* (UTEX LB 2063 and PCC 7806), and a genetically engineered microcystin synthetase knockout mutant of *M. aeruginosa* (PCC 7806 mcy$^-$, Dittmann et al. 1997) were each cultured semicontinuously in Cyano medium (20°C; illumination at 80 μmol quanta m^{-2} s^{-1}) at a dilution rate of 0.25 d^{-1} in aerated 5-liter vessels. *Cryptomonas* sp. (SAG 26.80) was grown in modified Woods Hole (WC) medium with vitamins (Guillard 1975) at 20°C with illumination at 120 μmol quanta m^{-2} s^{-1}. Carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (800 nm) and from carbon-extinction equations determined prior to each experiment.

Liposome preparation—Liposome stock suspensions were prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholin (POPC; Lipoid) dissolved in an aliquot of chloroform. Cholesterol- or EPA-containing liposomes were prepared by adding 3.33 mg of cholesterol or EPA (Sigma) from lipid stock solutions in chloroform. The resulting solutions were dried using a rotary evaporator, dissolved in 10 mL buffer (20 mmol L^{-1} NaP_i, 150 mmol L^{-1} NaCl, pH 7.0), and incubated on a rotary shaker (100 revolutions min^{-1}) for 30 min. Subsequently, the liposome suspensions were sonicated in an ultrasonic bath and excess free cholesterol and EPA were removed by washing the liposomes in fresh buffer using an ultraspeed centrifuge (150,000 $\times$ g, 90 min, 4°C). Prior to the addition of liposomes to the experimental beakers, the liposome stock suspensions were again sonicated (2 min). The liposome stock suspensions contained $\sim 1 \times 10^6$ liposomes mL^{-1} with a mean diameter of 4.4 μm. In a preliminary experiment, a dose-response curve was generated to test for possible effects of the increased availability of phospholipids and to assess the potential of the EPA- and cholesterol-containing liposomes to improve growth of *D. magna* (fed with 2 mg C of *S. elongatus*). The addition of increasing amounts of control and EPA-containing liposomes to the diet did not affect growth of *D. magna*. However, somatic growth rates increased with cholesterol availability. Cholesterol-saturated growth was obtained by adding 80 μL of the cholesterol-containing liposomes to the experimental beakers. Thus, 80 μL of a liposome stock suspension was used as a food supplement in each beaker. In treatments where cholesterol- and EPA-containing liposomes were supplemented simultaneously, 80 μl of each liposome stock suspension was used.

Daphnia growth experiments—Growth experiments were conducted with third-clutch juveniles (born within 8 h) of

a clone of *D. magna* (Lampert 1991). Stock cultures of *Daphnia* were raised in filtered lake water (0.2-μm pore-sized membrane filter) and saturating concentrations of *S. obliquus*. The growth experiments were carried out at 20°C in glass beakers filled with 200 mL of filtered lake water supplemented with 2 mg C L^{-1} of cyanobacterial carbon or *Cryptomonas* sp. (reference food). In one treatment, the cyanobacterial food suspensions were supplemented with 1 mg C L^{-1} of *S. elongatus* to test for a possible carbon limitation due to the inability of the animals to ingest filaments or cell colonies. Likewise, to analyze the role of grazing-resistant filaments in determining food quality, in one experiment filaments of *A. flos-aquae* (CCAP 1401-1) were mechanically disrupted using a mixer and subsequently fed to *D. magna*. Each treatment consisted of three replicates with six *D. magna* per beaker. Every day, animals were transferred into new beakers with freshly prepared food suspensions. All beakers were swirled four to six times a day to keep the cyanobacteria in suspension. Subsamples of the experimental animals were taken at the beginning and at the end of an experiment, dried for 24 h, and weighed on an electronic balance (Sartorius 4504MP8; ± 0.1 μg). The juvenile somatic growth rates (g) were determined as the increase in dry mass from day 0 (M_0) to day 6 (M_6) of the experimental period ($t = 6$ d) using the equation $g = (\ln M_6 - \ln M_0)\ t^{-1}$. Clutch sizes were estimated by counting the eggs in the brood chambers of the animals at the end of each experiment.

Analyses—For the analysis of fatty acids and sterols, approximately 0.5 mg particulate organic carbon (POC) was filtered separately onto precombusted GF/F filters (Whatman, 25 mm). The total lipids were extracted three times from filters with dichloromethane/methanol (2:1, v/v), and the pooled cell-free extracts were evaporated to dryness with nitrogen. The lipid extracts were transesterified with 3 mol L^{-1} methanolic HCl (60°C, 15 min) for the analysis of fatty acids or saponified with 0.2 mol L^{-1} methanolic KOH (70°C, 1 h) for the analysis of sterols. Subsequently, fatty acid methyl esters (FAMEs) were extracted three times with 2 mL of *iso*-hexan; the neutral lipids were partitioned into *iso*-hexan : diethyl ether (9 : 1, v/v). The lipid-containing fraction was evaporated to dryness under nitrogen and resuspended in a volume of 10 to 20 μL *iso*-hexan. Lipids were analyzed by gas chromatography on a HP 6890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-225 (J&W Scientific) capillary column to analyze FAMEs or with a HP-5 (Agilent) capillary column to analyze sterols. Details of GC configurations are given elsewhere (von Elert 2002 for fatty acids; Martin-Creuzburg and von Elert 2004 for sterols). Lipids were quantified by comparison with internal standards (C17:0 ME and C23:0 ME; 5α-Cholestan) using a flame ionization detector (FID) and identified by their retention times and their mass spectra, which were recorded with a gas chromatograph–mass spectrometer (Finnigan MAT GCQ) equipped with a fused-silica capillary column (DB-225MS, J&W for FAMEs; DB-5MS, Agilent for sterols). Sterols were analyzed in their free form and as their trimethylsilyl derivatives. Spectra were recorded between 50 and 600 amu in the electron impact (EI) ionization mode. The limit of quantitation was 20 ng for fatty acids or sterols. The absolute amount of each lipid was related to the particulate organic carbon (POC). Therefore, aliquots of the food suspensions were filtered onto precombusted glass-fiber filters (Whatman GF/F, 25-mm diameter) and analyzed for POC and nitrogen using an NCS-2500 analyzer (ThermoQuest GmbH). For determination of particulate phosphorus, aliquots were collected on acid-rinsed polysulfon filters (HT-200; Pall) and digested with a solution of 10% potassium peroxodisulfate and 1.5% sodium hydroxide for 60 min at 121°C, and soluble reactive phosphorus was determined using the molybdate-ascorbic acid method (Greenberg et al. 1985).

Table 1. Elemental nutrient ratios (molar) of *S. elongatus* (SAG 89.79), *A. variabilis* (ATCC 29413), *A. cylindrica* (SAG 1403-2), *A. flos-aquae* (CCAP 1401-1 and UTEX LB 2384), *M. aeruginosa* (UTEX LB 2063 and PCC 7806 mcy$^-$), and *Cryptomonas* sp. (SAG 26.80). Data are means of $n = 3$, $\pm$standard deviation (SD).

	C : N	C : P
S. elongatus (SAG 89.79)	4.3±0.1	62.9±2.9
A. variabilis (ATCC 29413)	4.6±0.1	79.1±3.4
A. cylindrica (SAG 1403-2)	4.7±0.1	60.4±0.9
A. flos-aquae (CCAP 1401-1)	4.6±0.1	140.5±8.5
A. flos-aquae (UTEX LB 2384)	4.8±0.1	195.8±1.6
M. aeruginosa (UTEX LB 2063)	4.7±0.0	61.8±0.7
M. aeruginosa (PCC 7806 mcy$^-$)	4.4±0.1	86.3±1.4
Cryptomonas sp. (SAG 26.80)	5.4±0.1	149.1±7.2

Data analysis—The somatic growth rates and clutch sizes of *D. magna* were analyzed using one-way analyses of variance (ANOVA). Treatments in which the animals did not produce eggs were excluded from the ANOVAs. ANOVAs were carried out using the general linear model module of Statistica 6.0 (StatSoft). Raw data met the assumption of homogeneity of variance; treatment effects were tested by Tukey's honestly significant difference (HSD) post hoc tests.

Results

Elemental and biochemical composition of the food sources—The molar carbon to nitrogen (C : N) and carbon to phosphorus (C : P) ratios of all cyanobacterial strains were low, indicating a high nitrogen and phosphorus content (Table 1). Thus, a limitation of *D. magna* by N or P is rather unlikely.

The fatty acid composition of *S. elongatus* was characterized by high amounts of short-chain saturated fatty acids, by the monounsaturated fatty acid 16:1n-7, and by the absence of PUFAs (Table 2). Strains of the genera *Anabaena* and *Aphanizomenon* contained considerable amounts of 18:2n-6 and, in particular, 18:3-n-3 (Table 2), but no PUFAs with more than 18 carbon atoms (cf. Gugger et al. 2002). Compared with the *Anabaena* and *Aphanizomenon* strains, the two *M. aeruginosa* strains contained high amounts of 18:3n-6 and 18:4n-3, but

comparatively small amounts of 18:3n-3 (Table 2). Both *Microcystis* strains did not contain PUFAs with more than 18 carbon atoms (cf. Gugger et al. 2002). The fatty acid composition of *Cryptomonas* sp. was dominated by high amounts of n-3 PUFAs, such as 18:3n-3, 18:4n-3, and 20:5n-3 (EPA).

Sterols were not detected in any of the cyanobacterial strains. Stigmasterol (24-ethylcholesta-5,22-dien-3β-ol; 4.2 $\pm$ 0.3 μg mg C^{-1}) and epibrassicasterol (24-methylcholesta-5,22-dien-3β-ol; 3.2 $\pm$ 0.1 μg mg C^{-1}) were the principal sterols found in *Cryptomonas* sp. (cf. Martin-Creuzburg et al. 2005*a*).

Elemental and biochemical characteristics of the prepared liposomes—Liposomes are a considerable source of carbon and phosphorus. A daily supply of 80 μL of the prepared liposome stock suspensions to the experimental beakers is equivalent to a supply of approximately 0.05–0.07 mg C d^{-1} (i.e., 12.5–17.5% of total carbon offered, depending on treatment) and 3.3 μg P d^{-1}. In a preliminary liposome dose-response test, a daily supply of up to 300 μL of the control liposome suspension to 200 mL lake water containing 2 mg C L^{-1} of *S. elongatus* did not affect *Daphnia* performance; i.e., the addition of liposomes per se had no beneficial or detrimental effect. The liposomes did not differ in their content of palmitic acid (16:0) and oleic acid (18:1n-9), which are both components of the phospholipids POPG and POPC (Table 3). Liposomes prepared in the presence of EPA contained considerable amounts of this fatty acid; liposomes prepared in the presence of cholesterol contained considerable amounts of this sterol, but neither EPA nor cholesterol were found in liposomes prepared without supplementing these compounds (Table 3).

Somatic growth rates and clutch sizes of D. magna—Juvenile somatic growth of *D. magna* on unsupplemented cyanobacteria was in general poor, and the animals did not produce eggs. In all experiments, growth rates and clutch sizes of *D. magna* were highest when grown on the reference food *Cryptomonas* sp. (Figs. 1–5). Irrespective of their lipid composition, the supplemented liposomes did not sustain positive growth rates of *D. magna* when offered without cyanobacterial carbon. In general, however, liposome-fed *D. magna* performed better than starving animals (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{4,9} = 16.54$, $p < 0.001$; Fig. 1).

Growth rates and clutch sizes on *S. elongatus* differed significantly between treatments (growth rates, ANOVA, $F_{6,14} = 1{,}163$, $p < 0.001$; clutch sizes, ANOVA, $F_{2,6} = 77.20$, $p < 0.001$; Fig. 2). Supplementation of *S. elongatus* with control liposomes or EPA-containing liposomes did not affect growth of *D. magna* (Tukey's HSD, $p > 0.43$). However, growth was significantly improved when *S. elongatus* was supplemented with cholesterol-containing liposomes, and growth was further enhanced by simultaneous supplementation with cholesterol- and EPA-containing liposomes (Tukey's HSD, $p < 0.05$). *D. magna* did not produce eggs when grown on unsupplemented *S. elongatus* or *S. elongatus* supplemented with control liposomes or EPA-containing liposomes (Fig. 2). Eggs were produced,

Table 2. Fatty acid composition of *S. elongatus* (SAG 89.79), *A. variabilis* (ATCC 29413), *A. cylindrica* (SAG 1403-2), *A. flos-aquae* (CCAP 1401-1 and UTEX LB 2384), *M. aeruginosa* (UTEX LB 2063 and PCC 7806 mcy^-), and *Cryptomonas* sp. (SAG 26.80). Data are expressed as μg mg C^{-1} ($n = 3$, $\pm$SD; n.d., not detectable).

Lipid	*S. elongatus* (SAG 89.79)	*A. variabilis* (ATCC 29413)	*A. cylindrica* (SAG 1403-2)	*A. flos-aquae* (CCAP 1401-1)	*A. flos-aquae* (UTEX LB 2384)	*M. aeruginosa* (UTEX LB 2063)	*M. aeruginosa* (PCC 7806 mcy^-)	*Cryptomonas* sp. (SAG 26.80)
16:0	25.69±0.55	33.17±1.04	42.38±0.46	35.16±3.33	60.97±0.59	45.46±0.90	51.16±0.92	22.11±0.98
16:1n-7	43.68±0.06	21.18±0.67	3.91±0.08	2.53±0.23	5.53±0.08	1.95±0.03	1.90±0.01	6.03±0.64
17:1n-7	0.69±0.01	1.26±0.03	n.d.	n.d.	n.d.	n.d.	n.d.	0.91±0.04
18:0	3.80±1.66	2.85±0.14	1.67±0.04	4.77±0.25	2.63±0.29	1.72±0.14	2.37±0.02	4.67±0.18
18:1n-9	1.15±0.19	6.96±0.14	5.94±0.03	4.99±0.25	6.66±0.17	0.86±0.09	2.01±0.06	4.92±0.21
18:1n-7	3.28±0.21	1.67±0.04	n.d.	1.58±0.06	0.93±0.02	0.62±0.01	1.35±0.04	8.04±0.28
18:2n-6	n.d.	12.52±0.34	15.62±0.07	9.49±0.13	16.72±0.38	4.29±0.02	11.28±0.27	17.39±0.22
18:3n-6	n.d.	n.d.	n.d.	n.d.	n.d.	30.56±0.27	33.14±0.77	0.91±0.06
18:3n-3	n.d.	42.59±1.19	53.34±1.23	66.85±0.54	93.00±2.20	11.03±0.20	5.88±0.14	75.30±0.58
18:4n-3	n.d.	1.57±0.10	n.d.	n.d.	n.d.	11.83±0.10	6.68±0.09	42.73±0.51
20:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.19±0.09
20:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.63±0.18
20:3n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.81±0.01
20:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	32.17±0.20
22:6n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.96±0.16

Table 3. Fatty acid and sterol composition of the liposomes used as a food supplement. Data indicate the amount that was supplied to *D. magna* with the different liposome suspensions (80 μL in 200 mL lake water, containing 2 mg cyanobacterial C L^{-1}); values are means of three replicates (n.d., not detectable). Data are means ±SD.

	Liposomes (control) (μg)	Liposomes + EPA (μg)	Liposomes + cholesterol (μg)
16:0	30.22±3.13	28.45±0.93	30.77±4.21
18:1n-9	29.79±1.94	28.94±1.76	29.60±2.70
20:5n-3	n.d.	34.17±0.83	n.d.
Cholesterol	n.d.	n.d.	15.85±0.42

however, when *S. elongatus* was supplemented with cholesterol-containing liposomes, and clutch sizes increased further by simultaneous supplementation with cholesterol- and EPA-containing liposomes (Tukey's HSD, $p < 0.05$).

Somatic growth of *D. magna* reared on *A. variabilis* was also significantly improved by cholesterol supplementation (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{6,14} = 413.63$, $p < 0.001$); simultaneous supplementation with cholesterol- and EPA-containing liposomes did not improve growth further (Fig. 3A). Supplementation of *A. variabilis* with control liposomes or EPA-containing liposomes did not affect somatic growth rates, and *D. magna* did not produce eggs when grown on unsupplemented *A. variabilis* or *A. variabilis* supplemented with control liposomes or EPA-containing liposomes (Fig. 3A). However, animals reared on cholesterol-supplemented *A. variabilis* produced eggs, and clutch sizes significantly increased by supplementing both cholesterol- and EPA-containing liposomes simultaneously (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{2,6} = 55.31$, $p < 0.001$). The addition of 1 mg C L^{-1} of *S. elongatus* to the *A. variabilis* food suspensions did not affect growth and reproduction of *D. magna*. A similar pattern was observed when *A. cylindrica* was used as food (Fig. 3B). Growth of *D. magna* on unsupplemented and EPA-supplemented *A. cylindrica* was poor, but it was significantly improved by cholesterol supplementation (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{6,14} = 292.44$, $p < 0.001$). Daphnids grown on *A. cylindrica* did not produce eggs (Fig. 3B).

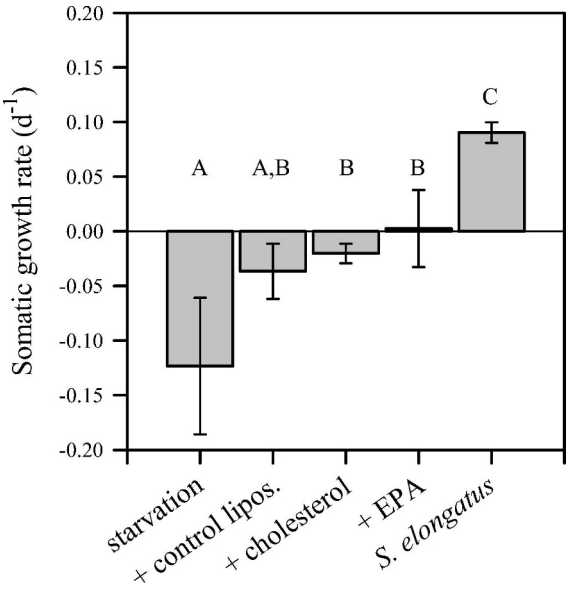

Fig. 1. Juvenile somatic growth rates of *D. magna* grown without food in filtered lake water (starvation), with control liposomes, with EPA- or cholesterol-containing liposomes, and with *S. elongatus*. Data are means of three replicates per treatment; error bars indicate standard deviation (SD). Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

Somatic growth rates of *D. magna* fed *A. flos-aquae* (CCAP 1401-1) were slightly increased by liposome supplementation, irrespective of the lipid composition of the liposomes, and by supplementation with *S. elongatus* (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{6,14} = 625.73$, $p < 0.001$; Fig. 4A). Liposome supplementation did not affect growth on *A. flos-aquae* strain UTEX LB 2384 (Tukey's HSD, $p > 0.10$ following ANOVA, $F_{6,14} = 321.97$, $p < 0.001$; Fig. 4B), whereas the addition of 1 mg C L^{-1} of *S. elongatus* to the *A. flos-aquae* food suspensions also marginally improved growth of *D. magna*. In contrast, growth of *D. magna* on mechanically disrupted filaments of *A. flos-aquae* (CCAP 1401-1) was significantly improved by cholesterol supplementation, while the supplementation with control liposomes or EPA-containing liposomes had no effect (Tukey's HSD following ANOVA, $F_{6,14} = 423.26$, $p < 0.001$; Fig. 4C). *D. magna* did not produce eggs when fed *A. flos-aquae* in either of the experiments (Fig. 4). The filament lengths of *A. variabilis*, *A. cylindrica*, and of the two *A. flos-aquae* strains are summarized in Table 4.

Somatic growth of *D. magna* reared on *M. aeruginosa* (UTEX LB 2063) was very poor (Fig. 5A). Supplementation of *M. aeruginosa* (UTEX LB 2063) with cholesterol marginally increased somatic growth rates of *D. magna* (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{6,14} = 715.21$, $p < 0.001$). Simultaneous supplementation with cholesterol- and EPA-containing liposomes did not further improve growth. Supplementation of the mcy^- mutant of PCC 7806 with EPA- or cholesterol-containing liposomes did not affect growth or reproduction of *D. magna* (Tukey's HSD, $p > 0.05$ following ANOVA, $F_{6,14} = 2{,}514.11$, $p < 0.001$; Fig. 5B). Growth rates on both *M. aeruginosa* strains were slightly increased by the addition of 1 mg C L^{-1} of *S. elongatus* to the food suspensions. The animals did not produce eggs when grown on either of the *M. aeruginosa* strains (Fig. 5). Animals reared on the microcystin-containing wild-type strain of PCC 7806 died within 4 d in all treatments (data not shown).

Growth of *D. magna* on a cyanobacterial mixture containing equal amounts of *S. elongatus*, *A. variabilis*, *A.*

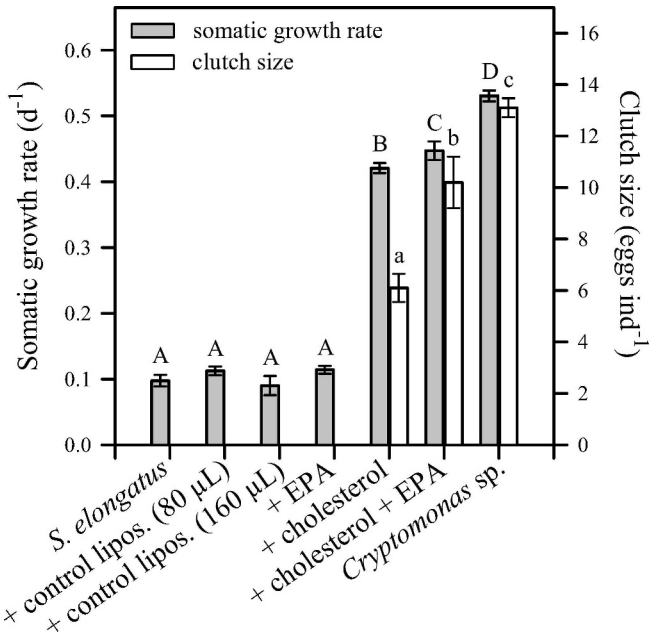

Fig. 2. Juvenile somatic growth rates and clutch sizes of *D. magna* grown on *S. elongatus* either unsupplemented or supplemented with and without EPA- and/or cholesterol-containing liposomes. Growth rates and clutch sizes on *Cryptomonas* sp. are shown for comparison. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

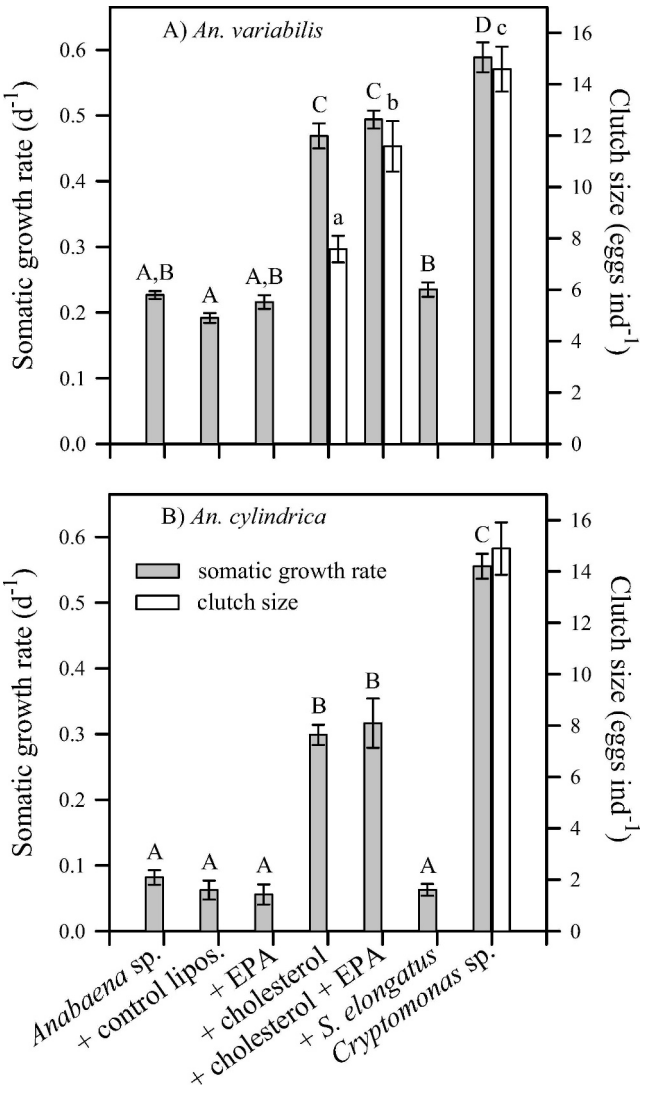

Fig. 3. Juvenile somatic growth rates and clutch sizes of *D. magna* grown on (A) *A. variabilis* and (B) *A. cylindrica* either unsupplemented or supplemented with and without EPA- and/or cholesterol-containing liposomes. Growth rates and clutch sizes on *Cryptomonas* sp. are shown for comparison. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

flos-aquae (CCAP 1401-1), and *M. aeruginosa* (UTEX LB 2384) (total 2 mg C) was significantly improved by cholesterol supplementation (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{6,14} = 649.24$, $p < 0.001$; Fig. 6). Simultaneously supplementing cholesterol- and EPA-containing liposomes did not further improve growth; clutch sizes, however, were significantly increased (Tukey's HSD, $p < 0.05$ following ANOVA, $F_{2,6} = 508.03$, $p < 0.001$; Fig. 6). Supplementation of 1 mg C L^{-1} of *S. elongatus* to the cyanobacterial mixture did not affect growth or reproduction of *D. magna*.

Discussion

It is well accepted that cyanobacteria are a nutritionally inadequate food source for *Daphnia* (e.g., Wilson et al. 2006), but the relative importance of the underlying mechanisms is still controversial. Here, we analyzed the role of sterols and EPA in determining the food quality of cyanobacteria for *D. magna* in order to integrate the role of essential lipids into the more classical theories of food-quality constraints caused by morphological properties or toxicity.

Growth on Synechococcus—The single-celled picocyanobacterium *S. elongatus*, which is nontoxic and well assimilated by *Daphnia* (Lampert 1977, 1981), has often been used to assess the significance of nutritive deficiencies in determining food quality for *Daphnia*. Previously, we have shown that somatic growth of daphnids (*Daphnia galeata*, *D. magna*) on *S. elongatus* is significantly improved by supplementation with cholesterol directly loaded onto the cyanobacterial cell, which demonstrated that the absence of sterols in *S. elongatus* accounts for the poor food quality of this cyanobacterium (von Elert et al. 2003; Martin-Creuzburg et al. 2005*b*). In contrast, however, Ravet et al. (2003) have found that growth of *D. pulex* on a cyanobacterial diet, consisting of *S. elongatus* and two *M. aeruginosa* strains, is improved by supplementation with EPA-containing liposomes, which would indicate a limitation by EPA rather than sterols. Here, to reassess the relative importance of sterols and EPA in determining the food quality of cyanobacteria for *Daphnia* and to rule out

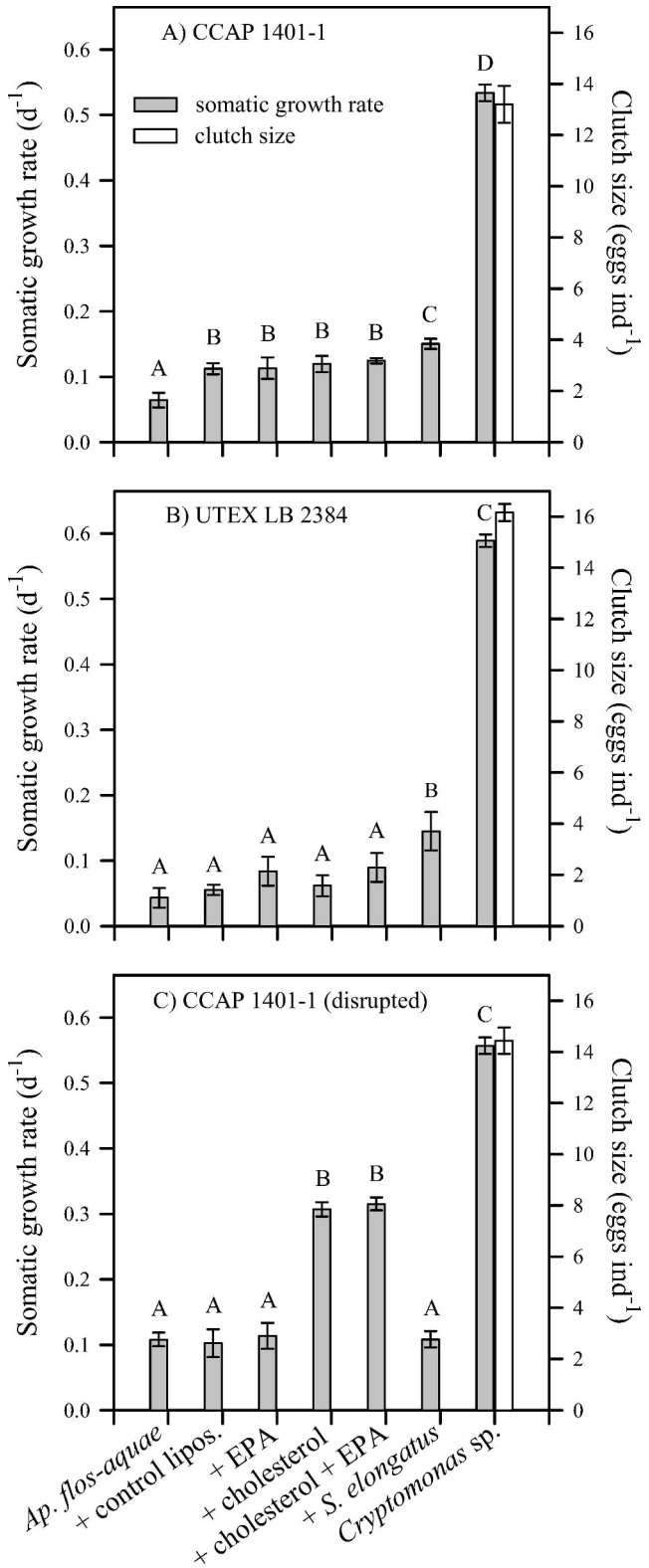

Fig. 4. Juvenile somatic growth rates and clutch sizes of *D. magna* grown on (A) *A. flos-aquae* (CCAP 1401-1) and (B) *A. flos-aquae* (UTEX LB 2384) either unsupplemented or supplemented with and without EPA- and/or cholesterol-containing liposomes. (C) Growth of *D. magna* on mechanically disrupted filaments of *A. flos-aquae* (CCAP 1401-1). Growth rates and clutch sizes on *Cryptomonas* sp. are shown for comparison. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

that the technique of lipid supplementation (direct loading onto the cell vs. liposomes) used in these studies accounted for these conflicting results, we used cholesterol- and/or EPA-containing liposomes as food supplements.

Supplementation of *S. elongatus* with cholesterol-containing liposomes considerably increased somatic growth rates of *D. magna* (from 0.10 to 0.42 d^{-1}), while supplementation with EPA-containing liposomes did not affect growth. Simultaneous supplementation of *S. elongatus* with cholesterol- and EPA-containing liposomes, however, further improved growth of *D. magna* and, more prominently, led to significantly increased clutch sizes compared with the cholesterol-supplemented treatment. This clearly indicates that growth of *D. magna* on *S. elongatus* is primarily limited by the absence of sterols and that EPA becomes limiting only when the shortage in sterols has been overcome by sterol supplementation and adds to the recent findings of von Elert et al. (2003) obtained with *D. galeata*. Hence, both methods of supplementation, i.e., liposomes and direct loading onto the cell, consistently demonstrated sterol limitation of *Daphnia* on a cyanobacterial diet. These results, however, are largely inconsistent with the findings of Ravet et al. (2003), who reported that somatic growth of *D. pulex* on a cyanobacterial mixture is primarily limited by the absence of EPA. The somatic growth rates of *D. pulex* on unsupplemented cyanobacteria presented by Ravet et al. (2003) were surprisingly high (up to approx. 0.42 d^{-1}) and clearly not in accordance with the widely accepted view of cyanobacteria as a low-quality food for *Daphnia* spp. Our finding that with sterol supplementation somatic growth rates increased to approximately 0.42 d^{-1}, a value obtained by Ravet et al. (2003) without supplementation, suggests that in the cyanobacterial cultures used by Ravet et al. (2003) sterols of unknown origin were present that released *D. pulex* from the sterol limitation. Unfortunately, in the study of Ravet et al. (2003) sterols were not determined; hence the origin of the putatively present sterols remains unclear. Summons et al. (2006) demonstrated that cyanobacterial cultures are easily contaminated by sterol-producing rust fungi, which provide an adequate source of sterols. Alternatively, sterols might have originated from the earth extract, which was used by Ravet et al. (2003) as a supplement in their cyanobacterial cultures. Here, we provide data showing that EPA becomes limiting only when the sterol requirements of the animals are met and demonstrate that this hierarchy in biochemical limitation holds for several cyanobacterial species in single cultures as well as in mixtures.

Unlike von Elert et al. (2003), we here assessed the effects of dietary lipids on somatic growth and egg production, since a limitation by dietary lipids might affect somatic growth and reproduction differently. It has been reported

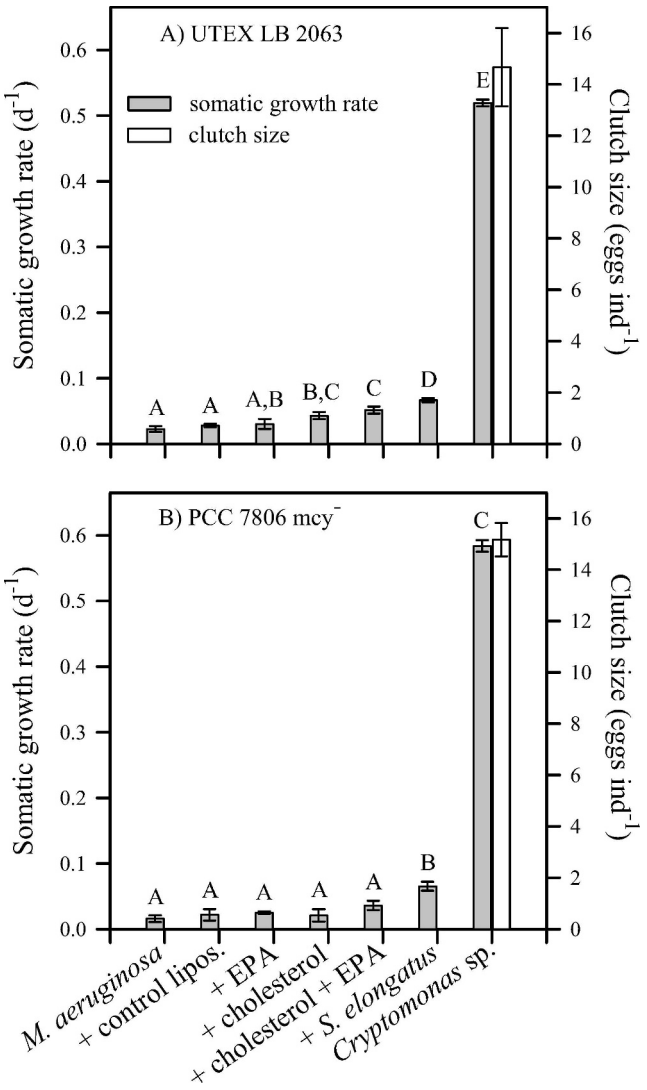

Fig. 5. Juvenile somatic growth rates and clutch sizes of *D. magna* grown on (A) *M. aeruginosa* (UTEX LB 2063) and on (B) the mcy$^-$ mutant of *M. aeruginosa* (PCC 7806 mcy$^-$) either unsupplemented or supplemented with and without EPA- and/or cholesterol-containing liposomes. Growth rate and clutch sizes on *Cryptomonas* sp. are shown for comparison. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

that dietary EPA is preferentially allocated into the eggs (Becker and Boersma 2005; Wacker and Martin-Creuzburg 2007), which suggests that the availability of EPA in the diet affects egg production in *D. magna* and confirms our present finding that clutch sizes are enhanced by EPA supplementation. In decapod crustaceans, the importance of dietary n-3 PUFAs for the development of female gonads has already been recognized (Harrison 1990).

In general, supplementation of *S. elongatus* with cholesterol-containing liposomes improved growth of daphnids more than supplementation with cholesterol that was loaded directly onto the cyanobacterial cell (cf. von Elert et al. 2003; Martin-Creuzburg et al. 2005*b*). This suggests that the phospholipids provided with the supplemented liposomes improve the bioavailability of cholesterol, possibly by accelerated absorption and/or transport of cholesterol from the gut to the tissues, and confirms the suitability of liposomes in delivering sterols to the herbivorous grazer. That liposomes are useful for delivering EPA to daphnids has been shown already by Ravet et al. (2003). It should be noted that liposomes are a considerable source of carbon and phosphorus and that the supplementation with liposomes will provide a grazer with extra energy. This has been shown in the control experiment, where liposome-fed *D. magna* performed better than starving animals (Fig. 1). However, the liposomes used in our study were unsuitable as a sole food source for *D. magna*; i.e., irrespective of the lipid composition they did not sustain positive growth rates of *D. magna* when offered without cyanobacterial carbon. This indicates that the effects of liposome supplementation presented here are directly related to the cyanobacterial diet and not caused by the liposome supplementation per se.

Table 4. Filament lengths of *A. variabilis* (ATCC 29413), *A. cylindrica* (SAG 1403-2), and *A. flos-aquae* (CCAP 1401-1 and UTEX LB 2384). Data are means of $n = 60$, ±SD.

Species	(μm)
A. variabilis (ATCC 29413)	595±222
A. cylindrica (SAG 1403-2)	481±149
A. flos-aquae (CCAP 1401-1)	291±104
A. flos-aquae (CCAP 1401-1)	118±53
Mechanically disrupted *A. flos-aquae* (UTEX LB 2384)	348±150

Growth on Anabaena *and* Aphanizomenon—The filamentous species of the genera *Anabaena* and *Aphanizomenon* belong to the most common bloom-forming cyanobacteria in freshwater ecosystems (Oliver and Ganf 2000). Mechanical interference with the filtering process of the cladocerans is regarded as the main reason for the poor assimilation of filamentous cyanobacteria (Porter and McDonough 1984; Gliwicz 1990). Here, however, we present data showing that growth of *D. magna* on *A. variabilis* and *A. cylindrica* is significantly improved by supplementation with cholesterol, which indicates sterol limitation as the major food-quality constraint, rather than mechanical interference. The simultaneous supplementation with cholesterol and EPA did not further improve growth of *D. magna* but led to significantly increased clutch sizes, at least when fed *A. variabilis*, which further corroborates the hypothesis that PUFAs play a crucial role in *Daphnia* reproduction.

The overall lower growth rates of *D. magna* fed *A. cylindrica*, and the inability of the animals to produce eggs within the 6-d experiment, may suggest that mechanical interference has hampered the assimilation of cyanobacterial carbon in the first hours or days of the experimental period when the animals were small and that the absence of sterols has limited growth in the following days when the animals became large enough to break up the *A. cylindrica* filaments and when maternally derived sterols were

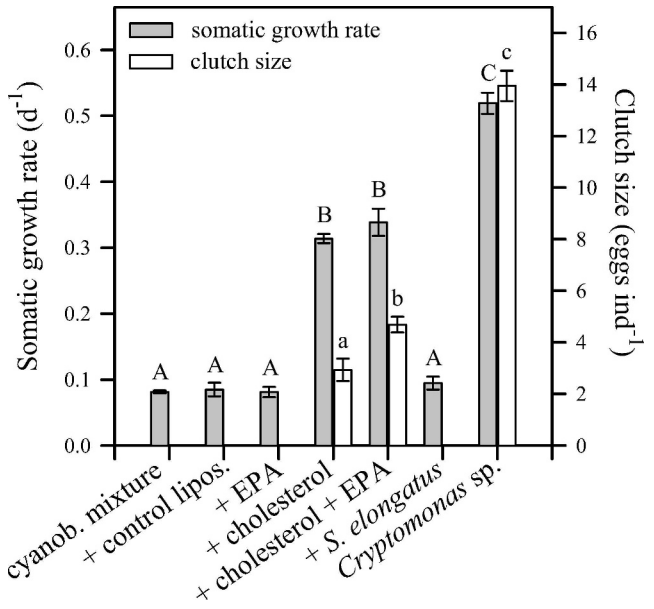

Fig. 6. Juvenile somatic growth rates and clutch sizes of *D. magna* grown on a cyanobacterial mixture containing equal amounts of carbon of *S. elongatus*, *A. variabilis*, *M. aeruginosa* (UTEX LB 2063), and *A. flos-aquae* (CCAP 1401-1) either unsupplemented or supplemented with and without EPA- and/or cholesterol-containing liposomes. Growth rate and clutch sizes on *Cryptomonas* sp. are shown for comparison. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

exhausted. It has been reported that the filtering rates of *D. pulex* feeding on *A. flos-aquae* filaments increase as a function of the body length of *D. pulex* (Holm et al. 1983), which would corroborate this hypothesis. Mechanical interference, however, cannot be the main reason for the poor food quality of *A. cylindrica*, since cholesterol supplementation clearly improved somatic growth, whereas the addition of 1 mg C L^{-1} of *S. elongatus* (i.e., the addition of ingestible carbon) to the *A. cylindrica* food suspensions had no effect. It is a general perception that daphnids can ingest particles in a size range of ~30–50 μm (Burns 1968). This, however, refers to beads, not to large filaments that might be destructed during the filtering process (Dawidowicz 1990). Microscopic analysis revealed that the guts of the animals were clearly filled with *A. variabilis* or *A. cylindrica*, respectively, which also indicates that *D. magna* is able to break up and manipulate the *Anabaena* filaments in a way that allows ingestion (*see* Table 4 for filament lengths).

In contrast, the supplementation of the *A. flos-aquae* strains with cholesterol did not affect growth of *D. magna*. Instead, growth on *A. flos-aquae* strain CCAP 1401-1 was slightly increased by liposome supplementation, irrespective of the lipid composition of the liposomes, which suggests that mechanical interference has hampered the carbon assimilation rather than the absence of essential lipids and that the addition of suitable carbon provided with the liposomes accounted for the significantly increased growth rates. This was supported by the finding that the addition of 1 mg C L^{-1} of *S. elongatus* to the *A. flos-aquae* (CCAP 1401-1) food suspension as well marginally improved growth of *D. magna*. This pattern was less clear, however, when *A. flos-aquae* strain UTEX LB 2384 was used as food, although the addition of 1 mg C L^{-1} of *S. elongatus* also significantly improved growth of *D. magna*. Microscopic analysis revealed that the filtering appendages of *D. magna* fed the *A. flos-aquae* strains were often blocked by large filament tufts and that the guts of the animals became brown and finally clear during the growth experiments, which suggests that the animals stopped feeding (cf. Holm and Shapiro 1984). Holm et al. (1983) reported that *D. pulex* is capable of grazing single filaments (200 μm long, broken from colonies) and small colonies (<1.5 mm long) of *A. flos-aquae*. In our experiments, the average length of single filaments was 291 μm for *A. flos-aquae* (CCAP 1401-1) and 348 μm for *A. flos-aquae* (UTEX LB 2384) (Table 4). In both strains aggregated filament tufts with a length of up to 1.8 mm were observed sporadically. To test for a possible carbon limitation due to the inability of the animals to ingest these large filaments, *D. magna* was fed *A. flos-aquae* (CCAP 1401-1) of which the filaments were mechanically disrupted to a mean size of 118 μm (Table 4). The disrupted *A. flos-aquae* filaments were readily ingested by *D. magna*, as indicated by an ample gut filling, and led to slightly increased somatic growth rates, as compared with the growth on nondisrupted filaments. Nevertheless, growth of *D. magna* on unsupplemented *A. flos-aquae* was poor. Supplementation with cholesterol, however, significantly improved somatic growth of *D. magna*, which indicates a shift from a limitation by energy (i.e., carbon) to a limitation by sterols with increased ingestibility of the *A. flos-aquae* filaments.

It has been suggested that the general inadequacy of *A. flos-aquae* to sustain growth and reproduction of *D. pulex* is due to a low assimilation efficiency (Holm et al. 1983; Holm and Shapiro 1984). This is largely inconsistent with our finding that growth of *D. magna* on mechanically disrupted filaments of *A. flos-aquae* was clearly enhanced by sterol supplementation, which suggests that *A. flos-aquae* is readily assimilated once it is ingested. However, the inability of the animals to produce eggs in this experiment may indicate that a low assimilation efficiency of *A. flos-aquae* has hampered to some degree the acquisition of carbon and lipids from the diet. Nevertheless, we conclude that the poor ingestibility of *A. flos-aquae* is the more important food-quality constraint for *D. magna* than the potential low assimilation efficiency.

The observed differences in the ability of *D. magna* to feed on the *Anabaena* and *Aphanizomenon* filaments might be due to different filament characteristics; i.e., filaments of *Anabaena* appeared to be more soft and flexible than the more rigid filaments of the *Aphanizomenon* strains. DeMott (1995) has already demonstrated that the hardness of a food particle strongly affects its ingestibility, especially for large particles that have to be manipulated prior to ingestion.

Although certain *Anabaena* and *Aphanizomenon* strains have been shown to produce a variety of toxins (Dow and Swoboda 2000), we did not find evidence for toxicity of the

Anabaena and *Aphanizomenon* strains used in our study to *D. magna*. In all experiments, *D. magna* performed better on either of these filamentous strains than when raised without food. Although this finding does not explicitly rule out that an unknown toxic compound has to some degree hampered the assimilation of cyanobacterial carbon and therewith the performance of *D. magna* (e.g., a feeding deterrent), we conclude that the nutritional deficiency of the *Anabaena* and also of the *Apanizomenon* strains is more important than the production of any toxic compound.

Growth on Microcystis—Toxin production is a characteristic feature of several strains of the unicellular cyanobacterium *M. aeruginosa*. A large variety of cyclic heptapeptides, termed microcystins, have been identified in *M. aeruginosa* that potentially cause hazards to human health and livestock (Carmichael 1994). In *Daphnia*, microcystins act as potential inhibitors of certain protein phosphatases, and the inhibition of these enzymes has been discussed as a mechanism involved in poisoning of *Daphnia* (DeMott and Dhawale 1995). Large amounts of microcystins have been found in the wild type of *M. aeruginosa* PCC 7806 (Dittmann et al. 1997). Using the genetically engineered microcystin-free mutant of PCC 7806, Rohrlack et al. (1999, 2001) have shown that the microcystins present in the wild type of PCC 7806 cause the death of daphnids. This finding was substantiated by our experiments where animals grown on the microcystin-containing wild type died rapidly (within 4 d), while animals grown on the microcystin-free mutant survived the experimental period (6 d). Despite the absence of microcystins, however, growth of *D. magna* on the mcy$^-$ mutant of PCC 7806 was poor irrespective of cholesterol and/or EPA supplementation. Likewise, growth on *M. aeruginosa* (UTEX LB 2063) did not provide convincing evidence for a limitation of *D. magna* by essential lipids. Microscopic examinations of the experimental animals revealed an accumulation of cyanobacterial cells in the mandibular region of the experimental animals and a poor gut filling when either of the *M. aeruginosa* strains was offered as food. Several strains of *M. aeruginosa* are known to form cell colonies that might interfere with the *Daphnia* ingestion process. However, mechanical interference can hardly explain the observed feeding inhibition, since we did not find significant colony formation in our *M. aeruginosa* cultures. This suggests that the poor food quality of microcystin-free *M. aeruginosa* strains is mainly due to an inhibition of the ingestion process by an as yet unknown toxic compound (Lampert 1981; Rohrlack et al. 2001; Lürling 2003) rather than to a nutritional deficiency in essential lipids.

Growth on a cyanobacterial mixture—Growth of the rather unselective filter feeder *D. magna* on a cyanobacterial mixture containing equal amounts of *S. elongatus*, *A. variabilis*, *A. flos-aquae* (CCAP 1401-1), and *M. aeruginosa* (UTEX LB 2063) was significantly improved by supplementation with cholesterol. This clearly indicates that the absence of sterols in cyanobacteria is a major food-quality constraint for *D. magna*, even if poorly ingestible (*A. flos-aquae*) or putatively toxic strains (*M. aeruginosa*) are present. The supplementation with EPA did not affect growth, but led to significantly increased clutch sizes once the animals were released from sterol limitation by simultaneously supplementing EPA and cholesterol, which again suggests that EPA plays a crucial role in *D. magna* reproduction.

The aim of the present study was to elucidate the relative importance of the factors that affect the carbon transfer efficiency at the cyanobacteria–*Daphnia* interface. On the basis of our data we propose the following order of significance: ingestibility > toxicity > sterols > PUFAs. Thus, the deficiency in essential lipids (i.e., sterols and PUFAs) in cyanobacteria will affect the performance of *Daphnia* only if the cyanobacterial carbon is readily ingested and nontoxic. Likewise, toxicity will exert its negative effect on the zooplankton only if the cyanobacterial carbon is ingested (cf. Lampert 1981). This, of course, does not apply to feeding deterrents and other toxins that are potentially released into the water. Although toxicity is a common feature of many cyanobacterial genera, including *Anabaena*, *Aphanizomenon*, and *Microcystis*, it varies significantly between species, strains, and even between clones of the same isolate. In the field, 44% to 67% of cyanobacterial blooms were found to be toxic (assessed by mouse bioassay; Dow and Swoboda 2000). As long as toxic cyanobacterial strains predominate, the absence of essential lipids or any other nutritional deficiency may hardly affect the performance of daphnids. However, as suggested by a recent meta-analysis of laboratory growth experiments, the overall role of toxin production in determining food quality of cyanobacteria for cladocerans may be overestimated, as compared with nutritional deficiencies and morphological aspects (Wilson et al. 2006).

Notwithstanding the widespread notion that mechanical interference of large cyanobacteria is a major constraint for population increase of *Daphnia* in eutrophic lakes, there is considerable evidence that *Daphnia* can sometimes exert large negative effects on relatively grazing-resistant cyanobacteria. In the absence of zooplanktivorous fish, the biomass of large-bodied *Daphnia* increases and the biomass of cyanobacteria often decreases (Vanni et al. 1990; Paterson et al. 2002; Sarnelle 2007). Experimental studies have demonstrated that such negative effects on cyanobacterial biomass constitute direct effects of *Daphnia* (Paterson et al. 2002; Sarnelle 2003, 2007), which implies that *Daphnia* was able to ingest a share of the cyanobacterial biomass that exceeded cyanobacterial production. Suppression of grazing-resistant cyanobacteria by *Daphnia* is counterintuitive, and it has been suggested that indirect mechanisms, e.g., *Daphnia*-mediated changes in nutrient ratios and light availability, diminish the competitive strength of cyanobacteria (Mackay and Elser 1998; Paterson et al. 2002).

Ingestibility of cyanobacterial carbon by *Daphnia* might be determined by filament rigidity and the formation of filament tufts, rather than by filament length per se, as suggested by the contrasting effects observed with *Anabaena* (long, soft filaments that are readily ingested) and *Aphanizomenon* (long, rigid filaments that are poorly

ingested). It has been proposed that filamentous cyanobacteria are most vulnerable to *Daphnia* grazing at the initial phase of a cyanobacterial bloom, as long as short single filaments predominate (Oliver and Ganf 2000; Chan et al. 2004). Thus, in the initial stage of a cyanobacterial bloom, the performance of the rather unelective filter feeder *Daphnia* might be primarily constrained by the absence of sterols, rather than by mechanical interference of filaments with the filtering process. Accordingly, in natural phytoplankton assemblages the degree of mechanical interference of filaments with the filtering process of daphnids might depend on (1) the cyanobacterial species composition and (2) the stage of the cyanobacterial bloom, assuming that the filament length, and in particular the formation of large filament tufts, increases with the age of a bloom.

To elucidate the relative importance of sterols and PUFAs in determining the food quality of cyanobacteria for *D. magna*, cyanobacterial carbon was supplemented with cholesterol- and/or EPA-containing liposomes. The results show that the growth of daphnids on cyanobacterial monocultures is clearly constrained by the absence of sterols, provided that the cyanobacterial carbon is ingestible and nontoxic. However, even on a cyanobacterial mixture containing poorly ingestible and putative toxic strains, the growth of *D. magna* was clearly limited by the absence of sterols, which emphasizes the significance of sterols as a possible food-quality constraint. The absence of certain PUFAs in cyanobacteria is of minor importance for somatic growth but potentially affects egg production in *D. magna*. The effect of sterol limitation might be most severe on juvenile daphnids, since they have a higher specific growth rate than older daphnids and therefore might require large amounts of sterols to (1) build up and maintain cell membranes and (2) synthesize ecdysteroids. With age, however, sterol requirements may gradually be reduced while the animals increase their relative investment in reproduction (Lynch et al. 1986) and consequently their requirements for certain PUFAs.

The seemingly variable ability of *Daphnia* to suppress cyanobacterial mass developments indicates that a mechanistic approach that takes into account the different mechanisms of cyanobacterial interference with *Daphnia* is needed to lead to a better predictability of food chain interactions. Here, for the first time, the integration of different aspects of cyanobacterial food quality into one hierarchical system has been attempted based on experiments. The proposed order of significance, ingestibility $>$ toxicity $>$ sterols $>$ PUFAs, provides a hierarchy of cyanobacterial constraints on *Daphnia* that will have to be tested under many aspects.

First, the different mechanisms of cyanobacterial interference result in fundamentally different types of resource limitation for *Daphnia*. A low ingestibility drives *Daphnia* into carbon (energy) limitation that can be relieved by any other type of ingestible carbon. On the other hand, if ingestible and nontoxic cyanobacterial carbon is available *Daphnia* will be subjected to sterol limitation that can be overcome only by eukaryotic carbon. Hence, the kind of resource *Daphnia* is competing for differs fundamentally for cyanobacteria of high or low ingestibility, and it remains to be tested how this affects zooplankton competition.

Second, the proposed hierarchy leads to predictions for the evolution of *Daphnia* populations. It predicts that ingestibility and toxicity are the major cyanobacterial food-quality constraints *Daphnia* has to cope with, and hence positive selection in *Daphnia* should favor genotypes that are less susceptible to these traits. Interestingly, local adaptation (Wilson and Hay 2007) and rapid microevolution in *Daphnia* to adapt to the presence of toxic *M. aeruginosa* in their natural food (Hairston et al. 1999) have been demonstrated recently. Furthermore, phenotypic plasticity in *Daphnia pulicaria* as adaptation to high biomasses of colonial and filamentous cyanobacteria and increased tolerance of *D. magna* after exposure to toxic cyanobacteria (Gustafsson and Hansson 2004) strongly suggest that low ingestibility and toxicity constitute selective pressures that shape the population genetics of *Daphnia* and partly release *Daphnia* from these constraints. On the contrary, the absence of de novo synthesis of sterols in all arthropods makes a selection for less sterol-dependent genotypes of *Daphnia* rather unlikely.

Third, the occurrence of low ingestibility or toxicity in cyanobacteria-dominated lakes is often believed to result from high grazing pressure by *Daphnia*, e.g. after food chain manipulations (Benndorf 1995; Hansson et al. 1998). It remains to be tested whether according to this reasoning the abovementioned microevolution in *Daphnia* can be observed only in lakes of low fish-mediated mortality and whether in cyanobacteria-dominated lakes with high predation by planktivorous fish *Daphnia* are more frequently sterol limited.

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