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Colimitation of a freshwater herbivore by sterols and polyunsaturated fatty acids

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Empirical data providing evidence for a colimitation of an herbivore by two or more essential nutrients are scarce, particularly in regard to biochemical resources. Here, a graphical model is presented, which describes the growth of an herbivore in a system with two potentially limiting resources. To verify this model, life-history experiments were conducted with the herbivore *Daphnia magna* feeding on the picocyanobacterium *Synechococcus elongatus*, which was supplemented with increasing amounts of cholesterol either in the presence or the absence of saturating amounts of eicosapentaenoic acid (EPA). For comparison, *D. magna* was raised on diets containing different proportions of *S. elongatus* and the cholesterol- and EPA-rich eukaryotic alga *Nannochloropsis limnetica*. Somatic and population growth of *D. magna* on a sterol- and EPA-deficient diet was initially constrained by the absence of sterols. With increased sterol availability, a colimitation by EPA became apparent and when the sterol requirements were met, the growth-limiting factor was shifted from a limitation by sterols to a limitation by EPA. These data imply that herbivores are frequently limited by two or more essential nutrients simultaneously. Hence, the concept of colimitation has to be incorporated into models assessing nutrient-limited growth kinetics of herbivores to accurately predict demographic changes and population dynamics.

Keywords: *Daphnia magna*; *Synechococcus elongatus*; *Nannochloropsis limnetica*; multiple resource limitation; cholesterol; eicosapentaenoic acid

1. INTRODUCTION

Liebig's law of the minimum, established in the nineteenth century to assess crop production in agriculture, states that primary production is limited by the nutrient in the shortest supply (relative to demand) and that, once the requirements of a crop for a single limiting nutrient are met by fertilization, another nutrient becomes limiting. This concept implies that only one nutrient can be limiting at a given time and that in a variable environment with changing nutrient availabilities, the actual limiting nutrient changes continually. However, a strict interpretation of Liebig's law, in terms of a single limiting nutrient, turns out to be difficult as primary production in terrestrial and aquatic (marine and freshwater) ecosystems is frequently limited by more than one nutrient simultaneously rather than sequentially (Davidson & Howarth 2007; Elser *et al.* 2007; Saito *et al.* 2008).

The principle of Liebig's law and the possibility of a simultaneous limitation by multiple factors (i.e. colimitation) have also been considered in models describing energetical (in terms of carbon), mineral and biochemical limited growth responses of herbivores (Muller *et al.* 2001; Grover 2003, 2004; Raubenheimer & Simpson 2004). However, empirical data providing evidence for colimitation of herbivores by two or more essential nutrients are scarce, particularly in regard to biochemical resources.

Here, we applied the concept of colimitation to the nutrition of a freshwater herbivore (*Daphnia magna*) to identify food components that interact in their effects on the performance of a consumer. Cladocerans of the genus *Daphnia* are key components of freshwater food webs with high ecological relevance; owing to their abundance and high grazing activity on the phytoplankton, they provide a crucial link between primary and secondary production. However, the carbon transfer efficiency across the phytoplankton—*Daphnia* interface is often constrained by the low availability of elemental (mainly phosphorus) or biochemical (e.g. essential lipids) nutrients. In recent years, food quality research has increasingly focused on biochemical nutrient requirements of *Daphnia* species. In particular, a dietary deficiency in essential lipids, i.e. polyunsaturated fatty acids (PUFAs) and sterols, has been shown to impair the performance of daphnids (Müller-Navarra *et al.* 2000; von Elert 2002; Martin-Creuzburg *et al.* 2005). Both PUFAs and sterols integral parts of cell membranes and serve as precursors for many bioactive molecules. The long-chain PUFAs arachidonic acid (ARA) and eicosapentaenoic acid (EPA), for instance, are precursors of eicosanoids, which are thought to be relevant in arthropod reproduction (Stanley-Samuelson 1994; Heckmann *et al.* 2008). Sterols, on the other hand, are precursors of steroid hormones, such as ecdysteroids, which are involved in the process of moulting (Grieneisen 1994; Martin-Creuzburg *et al.* 2007).

In previous studies, we demonstrated that somatic growth of daphnids on a PUFA- and sterol-free diet is primarily constrained by the absence of sterols and that EPA becomes limiting only when the shortage of sterols has been overcome by sterol supplementation (von Elert

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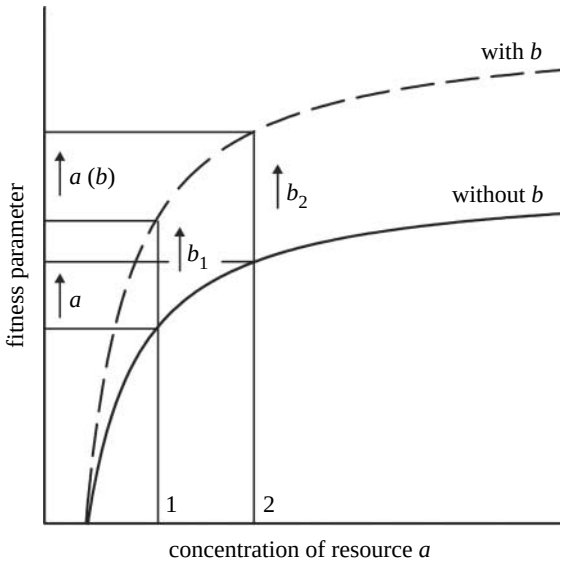

Figure 1. Hypothesized resource-dependent fitness in a system with two essential dietary resources (*a* and *b*) expressed as Monod curves. Fitness is improved with increasing concentrations of resource *a* in the diet following a saturation curve. If the concentration of resource *a* in the diet is increased from point 1 to 2, fitness is improved by *a* (*y*-axis) in the absence of resource *b* and by *a*(*b*) in the presence of resource *b*. At a given supply of resource *a*, e.g. at concentration 1 or 2 on the *x*-axis, fitness is improved by b_1 or b_2, respectively, when resource *b* is added to the diet. Thus, fitness is improved by the addition of both resources *a* and *b*, which indicates colimitation by these two essential resources (see text for more details).

et al. 2003; Martin-Creuzburg *et al.* 2008). However, this conclusion is based on the simple finding that the growth-enhancing effect of EPA becomes apparent only when surplus amounts of sterols (cholesterol) were supplied to exclude a limitation by sterols. With these data it is not possible to distinguish between a simple sequence of limiting nutrients where, according to Liebig's law, somatic growth is limited by the nutrient in the shortest supply, or a true colimitation where sterols and EPA affect somatic growth simultaneously (cp., Davidson & Howarth 2007). Here, to classify the type of limitation mediated by these two biochemical resources, a graphical model was developed, which describes the nutrient-limited growth of a herbivore in a system with two potentially limiting nutrients (figure 1). In this model, fitness (*y*-axis) is improved by *a* in the absence of resource *b* (e.g. EPA), and by *a*(*b*) in the presence of resource *b* when the concentration of resource *a* (*x*-axis, e.g. cholesterol) is increased from 1 to 2. At a given concentration of resource *a*, e.g. at 1 or 2 on the *x*-axis, fitness is improved by b_1 or b_2, respectively, when resource *b* is added to the diet. Hence, fitness can be improved by the addition of both nutrients *a* and *b*, which would indicate colimitation by these two essential resources.

To verify this model, we conducted life-history experiments with a clone of *D. magna* feeding on the sterol- and PUFA-free picocyanobacterium *Synechococcus elongatus*, which was supplemented with increasing amounts of cholesterol either in the presence or the absence of saturating amounts of EPA. The sterol-limited growth kinetics were analysed with regards to a possible colimitation of *D. magna* by sterols and EPA. For comparison, *D. magna* was raised on diets containing different proportions of *S. elongatus* and the cholesterol- and EPA-rich eukaryotic alga *Nannochloropsis limnetica*.

2. MATERIAL AND METHODS

(a) *Cultivation of food organisms*

The green alga *Scenedesmus obliquus* (SAG 276-3a) was used as food for daphnid stock cultures. 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, the cyanobacterium *S. elongatus* (SAG 89.79) and the eustigmatophyte *N. limnetica* (SAG 18.99) were each cultured semi-continuously in Cyano medium (20°C; illumination at 100 µmol quanta m^{-2} s^{-1}) at a dilution rate of 0.25 d^{-1} in aerated 5 l vessels. The coccoid *S. elongatus* was used because it is a non-toxic, phosphorus-rich cyanobacterium that is well-assimilated by *Daphnia* (Lampert 1977*a*,*b*, 1981), but lacks sterols and long-chain PUFAs (Martin-Creuzburg *et al.* 2008). *N. limnetica* was chosen because it contains high levels of cholesterol and EPA. Stock solutions of these food organisms were obtained by centrifugation and resuspension in fresh medium. Carbon concentrations of food suspensions were estimated from photometric light extinctions (800 nm) and from previously determined carbon-extinction equations.

(b) *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, Germany) dissolved in an aliquot of ethanol. Cholesterol- or EPA-containing liposomes were prepared by adding 3.33 mg cholesterol or EPA (Sigma) from lipid stock solutions in ethanol. 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. Excess free cholesterol and EPA were removed by washing the liposomes in fresh buffer using an ultra-speed centrifuge (150 000 *g*, 90 min, 4°C). Prior to the addition of liposomes to the experimental beakers, the liposome stock suspensions were sonicated again (2 min). The liposome stock suspensions contained approximately 1×10^6 liposomes ml^{-1} with a mean diameter of 4.2 µm.

(c) *Growth experiments*

Stock cultures of a clone of *D. magna* (Lampert 1991) were raised in filtered lake water (0.2 µm pore-sized membrane filter) with saturating concentrations of *S. obliquus*. Growth experiments were conducted with third-clutch juveniles (born within 8 h) at 20°C and a 16 : 8 h light:dark cycle in glass beakers filled with 200 ml of filtered lake water. Six food suspensions containing different proportions of *S. elongatus* ('Syn') and *N. limnetica* ('Nanno') were prepared: the total carbon concentration (2 mg C l^{-1}) consisted of 100 per cent Syn, 90 per cent Syn + 10 per cent Nanno, 80 per cent Syn + 20 per cent Nanno, 50 per cent Syn + 50 per cent Nanno, 20 per cent Syn + 80 per cent Nanno or 100 per cent Nanno. The cholesterol-supplemented diets were prepared by adding 5, 10, 20, 40, 80 or 100 µl of the cholesterol-containing liposome stock suspension to experimental beakers

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containing 100 per cent *S. elongatus*. Additional EPA supplementation was achieved by simultaneously adding 80 µl of the EPA-containing liposome stock suspension per beaker.

Each treatment consisted of three replicates with seven juvenile *D. magna* per beaker. Every day, the daphnids were transferred into new beakers with freshly prepared food suspensions. On the sixth day, three daphnids were subsampled, dried for 24 hour and weighed on an electronic balance (Mettler UMT 2; ± 0.1 µg). The juvenile somatic growth rates (g) were determined as the increase in dry mass from the beginning of an experiment (W_0) to day 6 (W_t) using the equation

$$g = \frac{\ln W_t - \ln W_0}{t}. \tag{3.1}$$

The remaining daphnids were kept in corresponding treatments until they had released their third-clutch juveniles. The number of viable offspring was determined in each successive reproduction cycle. Population growth rates (r) were estimated iteratively using the Euler–Lotka equation

$$1 = \sum_{x=0}^{n} l_x m_x e^{-rx}, \tag{3.2}$$

where l_x is the age-specific survivorship; m_x is the age-specific fecundity (number of neonates per individual); and x is the age at reproduction (in days). The probability of survival until reproduction (l_x) was estimated from the mortality that occurred in the different treatments. Growth rates were calculated as means of each treatment.

In preliminary growth experiments, *S. elongatus* was supplemented with 20, 80 and 180 µl of a liposome stock suspension prepared without cholesterol and EPA to test for the possible effects of the increased availability of phospholipids provided with the liposomes. The addition of these control liposomes to the diet did not affect the performance of *D. magna*, i.e. the addition of liposomes *per se* had no beneficial or detrimental effect. This indicated that the daphnids were not limited by the availability of carbon (food quantity) or phosphorus when grown on *S. elongatus*, because liposomes are a considerable source of these elements (cp., Martin-Creuzburg *et al.* 2008). Without simultaneously supplementing cholesterol, the addition of 80 µl of the EPA-containing liposome suspension to the diet also did not affect the growth of *D. magna* (cp., Martin-Creuzburg *et al.* 2008). Finally, the addition of 80 µl of the control liposome suspension to cholesterol-supplemented diets did not affect the kinetic of sterol-limited somatic or population growth. Whenever dietary cholesterol concentrations were calculated, the carbon content of the liposomes was considered.

(d) *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). Total lipids were extracted three times from filters with dichloromethane/methanol (2 : 1, v/v). Pooled cell-free extracts were evaporated to dryness using 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*-hexane; the neutral lipids were partitioned into *iso*-hexane:diethyl ether (9 : 1, v/v). The lipid-containing fraction was evaporated to dryness under nitrogen and resuspended in a volume of 10–20 µl *iso*-hexane. Lipids were analysed by gas chromatography on a HP 6890 GC equipped with a flame ionization detector (FID) and a DB-225 (J&W Scientific, 30 m × 0.25 mm ID × 0.25 µm film) capillary column to analyse FAMEs or with a HP-5 (Agilent, 30 m × 0.25 mm ID × 0.25 µm film) capillary column to analyse sterols. Details of GC configurations for the analysis of FAMEs are given elsewhere (von Elert 2002); sterols were analysed using the following configurations: oven, 150°C (1 min) to 280°C at 15°C min^{-1} then to 320°C at 2°C min^{-1}; carrier gas, helium (flow, 1.5 ml min^{-1}; velocity, 38 cm s^{-1}); detector, FID 350°C; injector, 350°C (total run time 30 min $sample^{-1}$). Lipids were quantified by comparison with internal standards (C17 : 0 ME and C23 : 0 ME; 5α-cholestan) of known concentrations, considering response factors determined previously with lipid standards purchased from Sigma or Steraloids. Lipids were 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; GC configurations as described for FID). Sterols were analysed in their free form and as their trimethylsilyl derivatives, which were prepared by incubating 20 µl of *iso*-hexane sterol extract with 10 µl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide including 1 per cent trimethylchlorosilane for 1 hour at room temperature. Spectra were recorded between 50 and 600 amu in the EI ionization mode. The limit for quantitation of fatty acids and sterols was 20 ng. The absolute amount of each lipid was related to the POC. Aliquots of food suspensions were therefore filtered onto precombusted glass fibre filters (Whatman GF/F, 25 mm diameter) and analysed for POC and nitrogen using an NCS-2500 analyzer (ThermoQuest GmbH, Egelsbach, Germany). For the determination of particulate phosphorus, aliquots were collected on acid-rinsed polysulphone filters (HT-200; Pall, Ann Arbor, MI, USA) and digested with a solution of 10 per cent potassium peroxodisulfate and 1.5 per cent sodium hydroxide for 60 min at 121°C. Soluble reactive phosphorus was determined using the molybdate–ascorbic acid method (Greenberg *et al.* 1985).

(e) *Data analysis*

The functional relationships between the dietary sterol content and the somatic (g) or population growth rates (r) were expressed as Monod curves (Monod 1950), modified with a threshold for zero growth (Rothhaupt 1988):

$$g = g_{\max}\frac{c - S_0}{c - S_0 + K_S} \quad r = r_{\max}\frac{c - S_0}{c - S_0 + K_S}, \tag{3.3}$$

where $g_{\max}$ and $r_{\max}$ are the maximum growth rates (d^{-1}); c is the resource concentration (µg mg C^{-1}); S_0 is the threshold concentration for zero growth (µg mg C^{-1}); and K_S is the half saturation constant (µg mg C^{-1}).

A nonlinear analogue to ANCOVA (Ratkowsky 1983; Wacker & von Elert 2001) was used to elicit changes in the nonlinear fit of the Monod model between *S. elongatus* supplemented with either cholesterol, or cholesterol and EPA, or *S. elongatus* mixed with *N. limnetica*. Datasets of somatic and population growth rates were analysed separately. For each of the three growth response curves (cholesterol, cholesterol and EPA, and *S. elongatus* mixed with *N. limnetica*) the residual sum of squares (RSS) of the

Monod model was determined. RSS were pooled to compare two individual datasets. Additionally, the RSS from a common model, which included the data from the two datasets, was computed. The significance of differences in the RSS from the common and the two individual models were then assessed by an *F*-test. This led to three comparisons between the three datasets. Nonlinear regression and the nonlinear analogue to ANCOVA after Ratkowsky (1983) were carried out using the statistical software package R v. 2.4.0, which is under general public licence (R Development Core Team, 2006).

The graphical approach used to describe colimitation of herbivore growth by two essential resources (figure 1) was also used to assess potential synergies between the two limiting nutrients, cholesterol and EPA. In this system, fitness should be improved by $a+b_1$ when the availability of resource a is increased from concentration 1 to concentration 2 and when resource b is added to the diet (figure 1). However, in our model, fitness is improved effectively by $a+b_2$, when the availability of resources a and b is increased simultaneously. To describe interacting effects of the two resources a and b on the growth of *D. magna*, we used the equation

$$I = (a + b_2) - (a + b_1) = b_2 - b_1.$$

In this equation, a positive interaction (I) indicates a synergistic effect of the two limiting resources a and b, i.e. fitness would be more improved by increasing the availability of both resources simultaneously than by increasing the availability of the two resources separately. A negative interaction indicates interfering effects of the two resources on the growth of the herbivore, e.g. by mutually restraining nutrient uptake.

Incipient limiting levels (ILLs) were estimated by comparing growth rates with one-way analysis of variance (ANOVA). The sterol concentration that led to a significant decrease in somatic or population growth rate with decreasing sterol supply was defined as ILL (cp., Martin-Creuzburg *et al.* 2005). A one-way ANOVA was also used to analyse the maximum numbers of offspring produced by *D. magna* within the first three reproduction cycles. ANOVAs were computed using the general linear model module of STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA). Raw data met the assumption of homogeneity of variance; treatment effects were tested by Tukey's HSD or Unequal N HSD *post hoc* tests. Significance levels of multiple tests used to assess ILLs were adjusted after Bonferroni (Rice 1989).

3. RESULTS

(a) *Biochemical composition of the food sources*

The molar carbon to nitrogen (C : N) and carbon to phosphorus (C : P) ratios of *S. elongatus* (means ± s.d.: C : N 4.0 ± 0.1; C : P 81.3 ± 0.9) and *N. limnetica* (C : N 5.4 ± 0.0; C : P 64.6 ± 1.4) were low, indicating a high nitrogen and phosphorus content. 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, the monounsaturated fatty acid 16 : 1*n*-7 and by the absence of PUFAs (table 1). By contrast, *N. limnetica* contained considerable amounts of 18 : 2*n*-6 and 20 : 4*n*-6, and exceptionally high amounts of 20 : 5*n*-3 (EPA; table 1). Sterols were not detected in *S. elongatus*. Cholesterol (cholest-5-en-3β-ol; 58.6% of total sterols), 24-ethylcholesterol (24-ethylcholest-5-en-3β-ol; 20.4%) and (iso)fucosterol (24*Z*/*E*-ethylidenecholesta-5,24(28)-dien-3β-ol; 21.0%) were the principal sterols found in *N. limnetica* (table 1). The C-24 stereochemistry of 24-ethylcholesterol and the *cis*/*trans* isomers fucosterol (*E*) and isofucosterol (*Z*) could not be identified with certainty. However, previous studies of sterols in freshwater eustigmatophytes suggested a 24β orientation of the ethyl group in 24-ethylcholesterol, and the occurrence of isofucosterol rather than fucosterol (Mercer *et al.* 1974; Volkman *et al.* 1999). It was assumed that these sterols can be converted to cholesterol by *D. magna* as has been shown by Martin-Creuzburg & von Elert (2004). Therefore, the growth response of *D. magna*, feeding on different mixtures of sterol-free *S. elongatus* and sterol-containing *N. limnetica*, was related to the total sterol content of the food suspensions, which was calculated by summing the individual amounts of the three principal sterols found in *N. limnetica* (table 1).

The prepared liposomes did not differ in their content of palmitic acid (16 : 0) and oleic acid (18 : 1*n*-9), which are both components of the phospholipids POPG and POPC (table 2). Liposomes prepared in the presence of

Table 1. Fatty acid and sterol composition of *S. elongatus* and *N. limnetica*; data represent means ± s.d. of three replicates (n.d., not detectable).

	S. elongatus (μg mg C^{-1})	*N. limnetica* (μg mg C^{-1})
14 : 0	13.57 ± 3.25	6.21 ± 0.40
14 : 1*n*-5	0.66 ± 0.44	n.d.
15 : 0	n.d.	2.54 ± 0.12
16 : 0	22.51 ± 1.87	23.93 ± 1.26
16 : 1*n*-7	34.84 ± 3.92	35.12 ± 2,22
18 : 0	2.65 ± 0.30	2.39 ± 0.02
18 : 1*n*-9/*n*-12	0.56 ± 0.38	3.31 ± 0.18
18 : 1*n*-7	2.37 ± 0.34	1.46 ± 0.07
18 : 2*n*-6	0.74 ± 0.85	4.59 ± 0.25
18 : 3*n*-6	n.d.	1.17 ± 0.06
18 : 3*n*-3	n.d.	1.92 ± 0.07
20 : 3*n*-6	n.d.	2.18 ± 0.16
20 : 4*n*-6	n.d.	9.79 ± 0.63
20 : 5*n*-3	n.d.	96.86 ± 5.81
total fatty acids	78.43 ± 6.67	193.04 ± 11.41
cholesterol	n.d.	6.59 ± 1.17
24-ethylcholesterol	n.d.	2.30 ± 0.18
(iso)fucosterol	n.d.	2.37 ± 0.35
total sterols		11.25 ± 1.63

Table 2. Fatty acid and sterol composition of the liposomes used as a food supplement. Data indicate the amount supplied to *D. magna* with 100 μl of the different liposome suspensions (100 μl in 200 ml lake water, containing 2 mg C l^{-1}); values are means ± s.d. of three replicates (n.d., not detectable).

	liposomes (control) (μg)	liposomes + cholesterol (μg)	liposomes + EPA (μg)
16 : 0	31.96 ± 0.15	28.07 ± 1.01	27.07 ± 1.56
18 : 1*n*-9	23.69 ± 1.71	21.08 ± 1.43	20.32 ± 1.42
20 : 5*n*-3 (EPA)	n.d.	n.d.	11.61 ± 1.28
cholesterol	n.d.	13.44 ± 0.43	n.d.

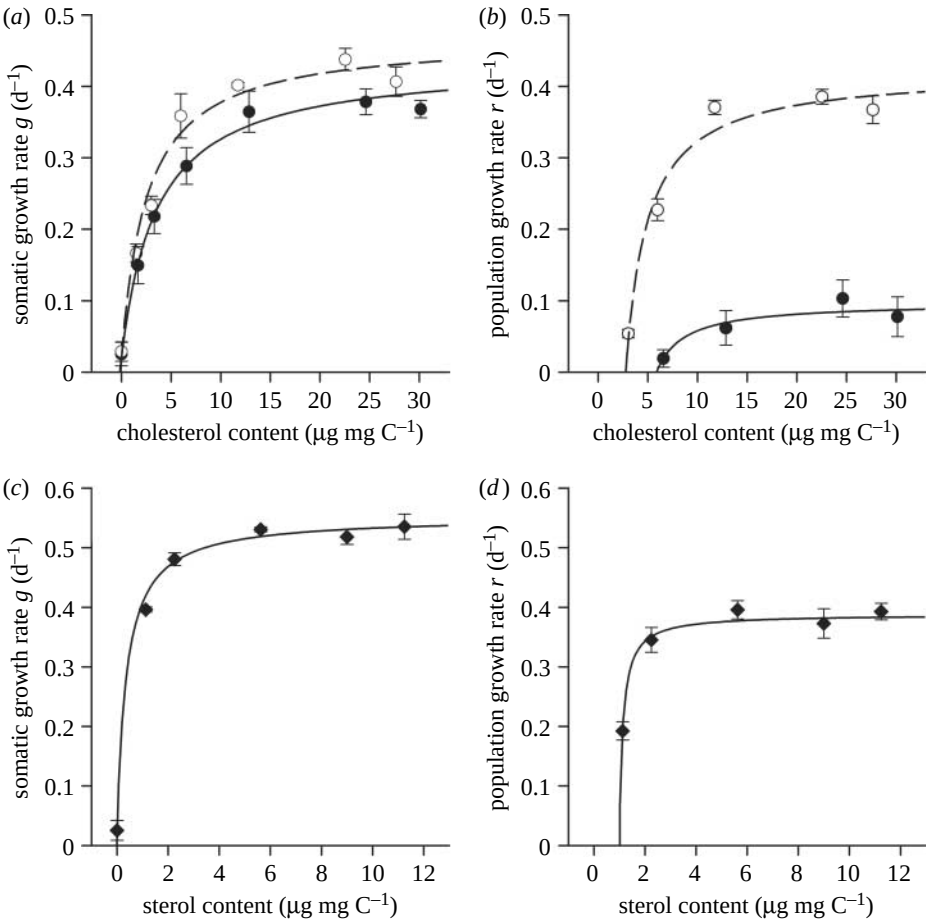

Figure 2. Growth kinetics of *D. magna* in response to the dietary sterol content. Somatic and population growth rates on cholesterol- and/or EPA-supplemented *S. elongatus* are shown in (*a*) and (*b*), respectively (filled circles, cholesterol; open circles, cholesterol + EPA). Somatic and population growth rates obtained using different mixtures of *S. elongatus* and the cholesterol- and EPA-rich *N. limnetica* are shown in (*c*) and (*d*), respectively (plotted against the total dietary sterol content; diamonds, *Synechococcus*/*Nannochloropsis*). The regression lines were calculated using a modified Monod model. Data are means of three replicates per treatment; error bars indicate standard deviation (s.d.).

Table 3. Comparisons between different Monod curves of somatic (*g*) and population growth (*r*) of *D. magna* using a nonlinear analogue to ANCOVA (Ratkowsky 1983). Datasets of somatic and population growth rates were analysed separately. Degrees of freedom of population growth were lower than that of somatic growth because *D. magna* did not reproduce at low dietary cholesterol concentrations.

	somatic growth (*g*)			population growth (*r*)		
	d.f.	*F*	*p*-value	*d.f.*	*F*	*p*-value
S. elongatus + cholesterol vs. *S. elongatus* + cholesterol + EPA	3.36	31.9	<0.001	3.21	4576	<0.001
S. elongatus + cholesterol vs. *S. elongatus*/*N. limnetica*	3.33	8100	<0.001	3.21	10738	<0.001
S. elongatus + cholesterol + EPA vs. *S. elongatus*/*N. limnetica*	3.33	2321	<0.001	3.24	2683	<0.001

cholesterol contained considerable amounts of this sterol, liposomes prepared in the presence of EPA contained considerable amounts of this fatty acid, but neither cholesterol nor EPA were found in liposomes prepared without supplementing these compounds (table 2).

(b) *Growth responses to sterol and EPA supply*

Somatic growth rates and estimated population growth rates of *D. magna* were highly correlated with the dietary sterol content, which was achieved either by supplementation of *S. elongatus* with cholesterol or by increasing the proportion of the (chole)sterol-rich alga *N. limnetica* in the diet (figure 2). Differences between the sterol-limited growth kinetics of *D. magna* obtained by supplementing *S. elongatus* with increasing amounts of cholesterol either in the absence or the presence of EPA were rather low for somatic growth but high for population growth (figure 2*a*,*b*). Statistical comparisons between the different Monod curves indicated that both somatic and population growth rates were significantly improved by additional EPA supplementation (table 3). As indicated by the missing overlap of the 95 per cent confidence intervals, the threshold concentration of cholesterol for positive population growth (S_0) was significantly higher than that for somatic growth (table 4). Moreover, the threshold concentration of cholesterol for positive population growth was significantly lower when cholesterol and EPA were supplemented simultaneously than when cholesterol

Table 4. Maximum somatic (g_{max}) and population (r_{max}) growth rates, threshold concentrations for zero growth (S_0) and half-saturation constants (K_S) for sterol-limited growth of *D. magna*. Animals were fed with the sterol-free picocyanobacterium *S. elongatus*, supplemented with increasing amounts of cholesterol, either in the presence or the absence of dietary EPA, or with different mixtures of *S. elongatus* with the cholesterol- and EPA-containing alga *N. limnetica* (95% confidence intervals in parentheses).

	somatic growth (g)			population growth (r)		
	g_{max} (d^{-1})	S_0 ($\mu g\ mg\ C^{-1}$)	K_S ($\mu g\ mg\ C^{-1}$)	r_{max} (d^{-1})	S_0 ($\mu g\ mg\ C^{-1}$)	K_S ($\mu g\ mg\ C^{-1}$)
S. elongatus+ cholesterol	0.435 (0.412–0.458)	−0.149 (−0.410–0.111)	3.391 (2.590–4.192)	0.097 (0.072–0.121)	5.876 (5.595–6.157)	2.786 (1.924–3.649)
S. elongatus+ cholesterol+ EPA	0.468 (0.441–0.494)	−0.079 (−0.299–0.140)	2.420 (1.842–2.999)	0.421 (0.393–0.449)	2.776 (2.548–3.004)	2.193 (1.593–2.793)
S. elongatus/ *N. limnetica*	0.552 (0.543–0.562)	−0.020 (−0.031–0.008)	0.400 (0.337–0.464)	0.388 (0.379–0.398)	0.991 (0.979–1.003)	0.133 (0.068–0.198)

was solely supplemented. By contrast, the threshold cholesterol concentrations for positive somatic growth did not differ (table 4).

The Monod curves obtained by feeding *D. magna* with cholesterol-supplemented *S. elongatus* differed significantly from those obtained by feeding *D. magna* with different proportions of *S. elongatus* and *N. limnetica* (table 3; figure 2*c*,*d*). Compared with the cholesterol-supplemented diets, the sterol-limited growth kinetics of *D. magna* obtained by increasing the proportion of *N. limnetica* in the diet were characterized by a higher slope of the regression line (i.e. a lower K_S), which suggests that a lower incremental increase in dietary sterols is required for the same increase in growth as was obtained by feeding *D. magna* with cholesterol-supplemented *S. elongatus* (figure 2). In contrast to somatic growth, the cholesterol threshold for positive population growth (S_0) was significantly lower for mixed algal diets than that for cholesterol-supplemented diets (table 4). The maximum somatic growth rates (g_{max}) were significantly higher for mixed algal diets than that for cholesterol-supplemented diets (irrespective of the presence or the absence of supplemented EPA), whereas the maximum population growth rates (r_{max}) were slightly lower for mixed algal diets than for cholesterol- and EPA-supplemented diets (overlap of confidence intervals approximately 1%; table 4).

Estimations of the ILL at which maximum growth passes into sterol-limited growth revealed that somatic and population growth rates of *D. magna* fed with different mixtures of *S. elongatus* and *N. limnetica* were significantly reduced at sterol levels of less than 5.6 $\mu g\ mg^{-1}$ of dietary carbon (comparisons of growth rates by one-way ANOVAs). Somatic and population growth rates of *D. magna* fed with cholesterol-supplemented *S. elongatus* were significantly reduced at sterol levels of less than 12.9 $\mu g\ mg^{-1}$ of dietary carbon, irrespective of EPA supplementation.

The maximum number of viable offspring produced by *D. magna* within the first three reproduction cycles was significantly affected by simultaneous EPA supplementation (Tukey's HSD test, $p<0.05$ following ANOVA, $F_{2,6}=113.70$, $p<0.001$; figure 3). In the absence of EPA, the number of offspring produced was generally low. The maximum number of offspring produced did not differ between *D. magna* feeding on cholesterol- and EPA-supplemented *S. elongatus* and those feeding on a mixture of *S. elongatus* and *N. limnetica* (figure 3).

Our data indicate that the growth of *D. magna* feeding on *S. elongatus* is primarily constrained by the absence of sterols. However, colimitation by sterols and EPA becomes apparent at a certain sterol supply, as indicated by the growth-enhancing effect due to cholesterol and EPA supplementation. To assess the potential synergistic effects of the two limiting nutrients, cholesterol and EPA, we applied the obtained growth response data to the graphical model presented in figure 1 and calculated how somatic and population growth rates were affected by the increased availability of cholesterol (resource *a*) in either the absence or the presence of EPA (resource *b*; see §2 for details). For example, if the availability of cholesterol is increased from 6 to 12 $\mu g\ mg\ C^{-1}$, the interaction (I) between cholesterol and EPA is close to zero ($-0.002\ d^{-1}$) for somatic growth, but slightly positive ($0.038\ d^{-1}$) for population growth, which suggests a synergistic effect of cholesterol and EPA supplementation on population growth.

4. DISCUSSION

We show here that somatic and population growth of the freshwater herbivore *D. magna* on a sterol- and PUFA-free diet (*S. elongatus*) is initially constrained by the absence of sterols, i.e. there is no growth response upon EPA supplementation without simultaneously supplementing cholesterol. With increased sterol availability, however, a colimitation by EPA becomes apparent, as indicated by the growth response upon EPA supplementation at a given sterol supply (cp., figures 1 and 2). This suggests colimitation of *D. magna* by sterols and EPA, once the availability of sterols enables (sterol-limited) growth. Finally, when the sterol requirements are met, the limiting factor for the growth of *D. magna* is shifted from sterols to EPA.

Differences between the sterol-limited growth kinetics obtained with and without simultaneously supplementing EPA were considerably more pronounced for population growth than for somatic growth (figure 2*a*,*b*), which indicates that a limitation by EPA gains importance in later life stages when the animals increase their investment in reproductive processes. This is in agreement with the finding that dietary EPA is preferentially allocated into

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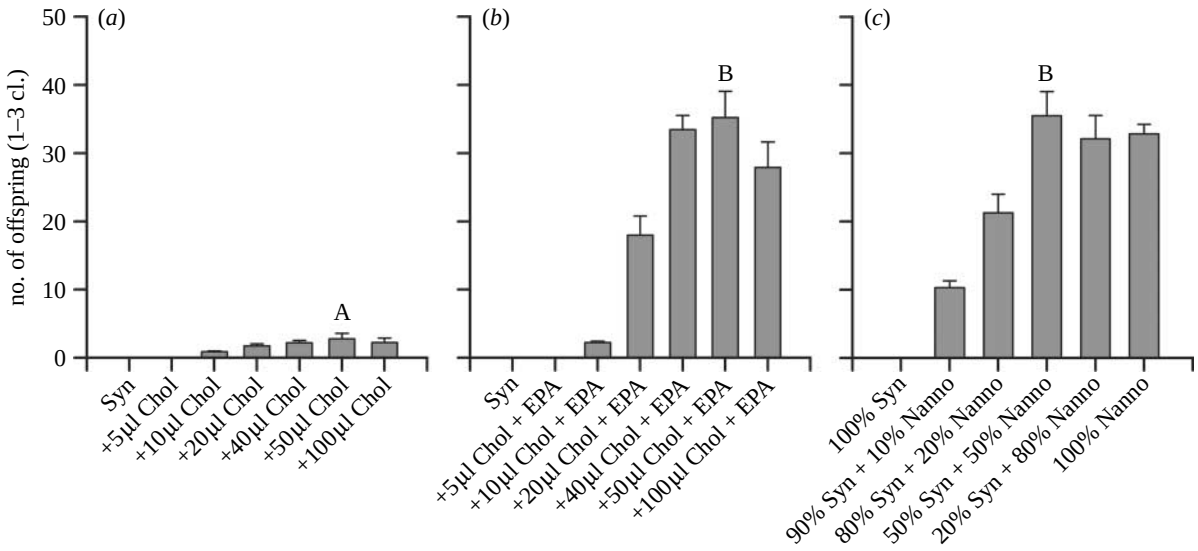

Figure 3. Number of viable offspring produced by *D. magna* within three reproduction cycles. Animals were fed with *S. elongatus* supplemented with increasing amounts of cholesterol-containing liposomes either in (*a*) the absence or (*b*) in the presence of EPA and with (*c*) different mixtures of *S. elongatus* and *N. limnetica*. Data are means of three replicates per treatment; error bars indicate s.d. Maximum values are indicated by upper case letters; bars labelled with the same letters are not significantly different (Tukey's HSD test, $p < 0.05$ following ANOVA).

the eggs by *D. magna* (Becker & Boersma 2005; Wacker & Martin-Creuzburg 2007), and corroborates the implication that EPA plays a crucial role in *Daphnia* reproduction (Becker & Boersma 2003; Martin-Creuzburg *et al.* 2008). Moreover, we show here that the total number of viable offspring produced by *D. magna* is significantly enhanced by EPA supplementation. Differential effects of food quality on growth and fecundity of *Daphnia* species have been reported previously (Urabe & Sterner 2001; Becker & Boersma 2003), which implies that life-table experiments, as in the present study, are required to reveal the potential effects of nutrient limitations rather than short-term growth experiments.

In principle, the sterol-limited growth kinetics obtained by feeding *D. magna* with sterol-supplemented *S. elongatus* were corroborated by feeding *D. magna* with different mixtures of *S. elongatus* and the cholesterol- and EPA-rich eukaryotic alga *N. limnetica*, i.e. somatic and population growth rates were also highly correlated with the dietary sterol content (figure 2*c*,*d*). However, in contrast to the cholesterol-supplemented diets, the Monod curves obtained by feeding *D. magna* with increasing proportions of *N. limnetica* were characterized by a higher incremental increase in somatic and population growth rates (slope of the regression line; lower K_S), which indicates that a lower dietary sterol content is required for the same increase in growth as is obtained with the cholesterol-supplemented diets. Differences between the growth response of *D. magna* obtained with the mixed algal and cholesterol-supplemented diets were also manifested in differences between the estimated ILLs at which maximum growth passes into sterol-limited growth. The sterol-limited growth response of *D. magna* obtained by increasing the relative proportion of *N. limnetica* in the diet suggested that somatic and population growth of *D. magna* is reduced at sterol levels of less than 5.6 µg mg^{-1} of dietary carbon. By feeding *D. magna* with different mixtures of *S. elongatus* and the green alga *S. obliquus*, we had previously suggested that somatic growth of *D. magna* is reduced, when the sterol content falls below 5.4 µg mg^{-1} of dietary carbon (Martin-Creuzburg *et al.* 2005), which is now corroborated by the present finding. By contrast, the sterol-limited growth response of *D. magna*, obtained by the supplementation of *S. elongatus* with increasing amounts of cholesterol, suggests that 12.9 µg of sterol per mg of dietary carbon are required to release *D. magna* from sterol limitation. These differences in the growth response obtained using mixed algal diets and cholesterol-supplemented diets might be due to differences in the dietary sterol composition. Although cholesterol was found to be the principal sterol in *N. limnetica*, two other sterols were detected, 24-ethylcholesterol and (iso)fucosterol. In a previous study, we showed that the growth of *Daphnia galeata* consuming *S. elongatus* is significantly more improved by supplementation with 24-ethylcholesterol (sitosterol, i.e. 24α-ethylcholest-5-en-3β-ol) than by supplementation with cholesterol (Martin-Creuzburg & von Elert 2004), which suggests a higher assimilation efficiency for 24-ethylcholesterol than for cholesterol. Thus, the higher incremental increase in somatic and population growth rates and the lower saturation thresholds obtained with the mixed algal diets might be due to a more suitable sterol composition of *N. limnetica* compared with the intrinsically sterol-free *S. elongatus*, which was supplemented solely with cholesterol. However, complex synergies with sterols and other potentially limiting nutrients present in *N. limnetica* and absent in *S. elongatus* can also not be excluded. Nevertheless, the maximum population growth rates obtained by feeding *D. magna* with cholesterol- and EPA-supplemented *S. elongatus* exceeded that obtained by feeding *D. magna* with the mixed algal diets (the confidence intervals overlapped only slightly; table 4), which indicated that the poor food quality of *S. elongatus* can be improved by cholesterol and EPA supplementation at least to match the level of *N. limnetica*.

In a recent review, Saito *et al.* (2008) differentiated between three types of colimitation of primary production in aquatic environments: type I, colimitation by

independent nutrients, which do not share a specific biochemical function (e.g. N and P); type II, colimitation by nutrients, which can substitute for the same biochemical function (e.g. Zn and Co); and type III, colimitation by biochemically dependent nutrients, where the ability to acquire one nutrient is dependent on sufficient supply of another (e.g. C and Zn). This classification system may help to improve our understanding of how primary production is affected by the availability of simultaneously limiting nutrients, but whether or not this concept is also applicable to the nutrient-limited growth of herbivores remains open. With regards to our data, the colimitation of *D. magna* by sterols and EPA can be considered as a type I colimitation (multiplicative form; for details see, Saito *et al.* (2008)), because sterols and PUFAs are presumably biochemically independent nutrients, although in part they have related functions in membrane physiology (e.g. both are involved in the regulation of membrane fluidity and permeability) and may interact as sterol esters in transport and storage of lipid resources. Instead, our data suggest that the availability of sterols and EPA affects different physiological processes, which are temporally connected to (dependent on) each other. Sterols are indispensable structural components of cell membranes and they serve as precursors for the moult-inducing ecdysteroids (Grieneisen 1994; Martin-Creuzburg *et al.* 2007), which suggest that they are primarily important for somatic growth. Outside of their structural role in cell membranes, the C-20 PUFAs EPA and ARA serve as precursors for eicosanoids, cell signalling molecules which are thought to be relevant in arthropod reproduction (Stanley-Samuelson 1994; Heckmann *et al.* 2008). Thus, once the sterol supply allows sterol-limited somatic growth and once an animal starts to allocate resources towards reproductive tissues (e.g. gonads, eggs), a (co)limitation by EPA becomes apparent. Hence, sterols and PUFAs can be considered as independent nutrients, where somatic growth is limited by the availability of sterols and reproduction is limited by the availability of EPA. However, it is unlikely that the availability of sterols solely affects somatic growth and the availability of EPA solely affects reproductive processes. Somatic growth rates, as presented in figure 2, are usually calculated using the increase in body dry mass during the experiment, which includes the mass increase due to the development of reproduction-related structures (e.g. gonads). In addition, to estimate how reproduction-related structures have contributed to the increase in body dry mass, we used the increase in body size (i.e. body length) to calculate somatic growth rates (data not shown). However, both ways of calculating growth rates revealed an increase in somatic growth when cholesterol and EPA were supplemented simultaneously compared with cholesterol supplementation alone, which indicated that somatic growth was also affected by the availability of EPA. Likewise, the availability of sterols will, to some extent, also affect reproduction, as was previously demonstrated for daphnids and also for copepods (Hassett 2004; Martin-Creuzburg & von Elert 2004; Martin-Creuzburg *et al.* 2005). It has been shown that daphnids allocate dietary sterols into their eggs presumably to provide the developing embryo with sufficient amounts of sterols (Wacker & Martin-Creuzburg 2007). Moreover, a gonadotrophic role of ecdysteroids has been suggested (Martin-Creuzburg *et al.* 2007), which implies that sterols are needed for ovarian maturation and egg production. In copepods, the dietary sterol content was found to affect not only egg production rates, but also egg viability (Crockett & Hassett 2005). Finally, the data presented here suggest that population growth of *D. magna* is improved more by the addition of sterols and EPA together than by adding either one nutrient separately, which implies a synergistic effect of sterol and EPA supplementation on reproduction. Nevertheless, we show here in *D. magna* that somatic growth is primarily constrained by the availability of sterols and reproduction is primarily constrained by the availability of EPA.

Saito *et al.* (2008) have already argued that, for primary production, the classification of different types of colimitation into the proposed three categories is complicated by the fact that many nutrients can be categorized into multiple types of colimitation. For instance, a limitation by nitrogen and phosphorus can be considered as a type I colimitation, while a limitation by nitrogen on urea and nickel (required for the metalloenzyme urease) can be considered as a type III colimitation (for details see, Saito *et al.* 2008). Here, with regards to the nutrient-limited growth kinetics of a herbivore, we add another complicating factor, which may hamper the clear classification of a colimitation into one of these three categories: a temporal change of limiting nutrients during ontogenetic development. In spite of these difficulties, the classification of different types of colimitations into distinct categories, as suggested by Saito *et al.* (2008), provides a basis for discussing the concept of colimitation with regards to both primary production and nutrient-limited growth of herbivores.

Our data imply that herbivores, such as *D. magna*, are frequently limited by two or more essential nutrients simultaneously. Thus, the concept of colimitation has to be incorporated into models assessing nutrient-limited growth kinetics of consumers to accurately predict demographic changes and population dynamics. Hitherto, most consumer-resource models rely on Liebig's law, i.e. they assume threshold functions that switch abruptly between limiting nutrients. Only recently, concepts based on synthesizing units combined with the dynamic energy budget model (e.g. Muller *et al.* 2001; Kooijman *et al.* 2004) have been used to describe the functional relationships between the assimilation and allocation of multiple elements and their possible effects on growth. The experimental set-up we presented here, using sterol- and/or EPA-limited *D. magna* as a model system, provides a promising tool to study nutrient-limited growth responses of an herbivore and to evaluate existing stoichiometric and biochemical approaches (e.g. Sterner & Elser 2002; Anderson *et al.* 2004) and more complex models of multiple resource limitations (Muller *et al.* 2001; Grover 2003, 2004; Raubenheimer & Simpson 2004; Saito *et al.* 2008). However, the development of a multidimensional model requires further studies in which not only the dietary sterol concentration is varied, but also the dietary EPA concentration (and/or other potentially limiting nutrients). It also has to be tested whether or not data obtained with this model system can be implemented into the geometrical framework developed by Raubenheimer & Simpson (2004) and references therein. This would require to sharpen the

focus on interactive effects of food components on organismal nutrition, i.e. on the way nutrients are manipulated to meet requirements (ingestive regulation, post-ingestive processing, etc.). In doing so, it has to be considered that daphnids are unselective filter feeders (DeMott 1986), i.e. unlike many terrestrial herbivores they presumably do not have the ability to equilibrate a dietary mismatch by compensatory feeding or selection of complementary food sources. However, daphnids might respond post-ingestively by excreting excess dietary compounds, which might be associated with metabolic costs (Anderson *et al.* 2005).

In general, understanding the trophic interactions and food web regulation and their response to altered nutrient and climate conditions demands more information concerning the concept of colimitation from empirical and theoretical studies. This would highly improve our understanding of the complex interplay between energetic, mineral (e.g. phosphorus) and biochemical (e.g. fatty acids and sterols) limitations of herbivores.

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