Biochemical nutrient requirements of the rotifer *Brachionus calyciflorus*: co-limitation by sterols and amino acids

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Summary

1. It has been proposed that growth and reproduction of animals is frequently limited by multiple nutrients simultaneously. To improve our understanding of the consequences of multiple nutrient limitations (i.e. co-limitation) for the performance of animals, we conducted standardized population growth experiments using an important aquatic consumer, the rotifer *Brachionus calyciflorus*.

2. We compared nutrient profiles (sterols, fatty acids and amino acids) of rotifers and their diets to reveal consumer–diet imbalances and thus potentially limiting nutrients. In concomitant growth experiments, we directly supplemented potentially limiting substances (sterols, fatty acids, amino acids) to a nutrient-deficient diet, the cyanobacterium *Synechococcus elongatus*, and recorded population growth rates.

3. The results from the supplementation experiments corroborated the nutrient limitations predicted by assessing consumer–diet imbalances, but provided more detailed information on co-limiting nutrients. While the fatty acid deficiency of the cyanobacterium appeared to be of minor importance, the addition of both cholesterol and certain amino acids (leucine and isoleucine) improved population growth rates of rotifers, indicating a simultaneous limitation by sterols and amino acids.

4. Our results add to growing evidence that consumers frequently face multiple nutrient limitations and suggest that the concept of co-limitation has to be considered in studies assessing nutrient-limited growth responses of consumers.

Key-words: consumer, consumer–diet imbalance, dietary mismatch, fatty acid, global change, lipid, nutrition, phytoplankton, tetrahymanol, zooplankton

Introduction

The concept of co-limitation, that is, a simultaneous limitation by multiple nutrients, has been frequently used to describe nutrient-limited growth responses of primary producers (Davidson & Howarth 2007; Elser *et al.* 2007; Saito, Goepfert & Ritt 2008; Harpole *et al.* 2011). As yet, however, it has been poorly applied to the nutrient-limited growth of animals (Martin-Creuzburg, Sperfeld & Wacker 2009; Lukas, Sperfeld & Wacker 2011; Sperfeld, Martin-Creuzburg & Wacker 2012), which besides elemental nutrients also rely on a number of essential biochemicals (e.g. Beenakkers, Van der Horst & Van Marrewijk 1985). As

yet, experiments providing clear evidence for a co-limitation of an animal by two or more essential nutrients are scarce, in particular in regard to biochemical nutrients. Recently, using the freshwater herbivore *Daphnia* as a model organism, we have shown that the growth of this herbivore can be limited simultaneously by a low availability of dietary sterols and polyunsaturated fatty acids (PUFAs) (Martin-Creuzburg, Sperfeld & Wacker 2009; Sperfeld, Martin-Creuzburg & Wacker 2012). Both sterols and PUFAs are indispensable structural components of cell membranes (Hulbert & Else 2005; Hulbert *et al.* 2005) and both serve as precursors for a number of bioactive molecules. Sterols, for instance, are precursors of steroid hormones, such as ecdysteroids, which in arthropods are involved in the process of moulting (Grieneisen 1994). Long-chain PUFAs, such as arachidonic acid (ARA) and

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eicosapentaenoic acid (EPA), are precursors of eicosanoids, which are thought to be relevant in invertebrate reproduction, the immune system and other physiological processes (Stanley-Samuelson 1994; Heckmann *et al.* 2008).

In aquatic ecosystems, a low availability of essential lipids and consequently a lipid limitation of zooplankton has been supposed to be associated with a high share of cyanobacteria within the phytoplankton (Müller-Navarra *et al.* 2000; Von Elert, Martin-Creuzburg & Le Coz 2003), because cyanobacteria lack sterols and long-chain PUFAs (> C18) (Volkman 2003; Summons *et al.* 2006; Martin-Creuzburg, Von Elert & Hoffmann 2008). As cyanobacterial bloom formation, with all its negative effects on water quality, is presumably favoured by global warming (Jöhnk *et al.* 2008; Paerl & Huisman 2008), a limitation of zooplankton consumers by essential lipids may be intensified in near future, in particular in eutrophic systems (Sperfeld & Wacker 2009).

In contrast to fatty acids and sterols, the relevance of amino acids as potentially limiting nutrients has been mostly neglected in food quality studies. However, it has been shown that the availability of certain dietary amino acids correlates with the fecundity and hatching success of marine copepods (Guisande, Maneiro & Riveiro 1999; Guisande, Riveiro & Maneiro 2000; Helland *et al.* 2003) and a potential limitation of copepods by a low availability of the amino acid arginine has been suggested by Anderson, Boersma & Raubenheimer (2004) based on comparisons between amino acid profiles of copepods and their food. Moreover, it has been suggested that specific dietary amino acids, in particular arginine and histidine, are involved in triggering the switch between reproductive modes in *Daphnia*, that is, they have been found to avert crowding-induced resting egg production and to enhance subitaneous reproduction (Koch *et al.* 2011). As amino acid profiles of natural food sources vary seasonally, (Cowie & Hedges 1992; Petersson & Floderus 2001; Kalachova *et al.* 2004), the performance of freshwater herbivores might be constrained not only by a low availability of essential dietary lipids, but also by the availability of certain amino acids, which potentially results in a simultaneous limitation by multiple nutrients.

Rotifers are important components of freshwater food webs (Walz 1995) that can dominate the zooplankton with densities often >1000 individuals per litre (Modenutti 1994; Dagne *et al.* 2008). By feeding on bacteria, algae, heterotrophic nanoflagellates and ciliates (Pourriot 1977; Sanders *et al.* 1989; Arndt 1993) and by serving as food, for example, for copepods and the larvae of insects and fishes, they are significantly involved in transferring energy and nutrients to higher trophic levels (Williamson 1983; Walsh 1995; Walz 1995). However, the efficiency with which rotifers use assimilated carbon for biomass production is potentially constrained by a low availability of essential nutrients, that is, elements (Rothhaupt 1995; Urabe, Clasen & Sterner 1997; Jensen & Verschoor 2004) or PUFAs

(Weithoff & Wacker 2007; Wacker & Weithoff 2009). Thus, given their high ecological relevance for food web processes rotifers are, besides *Daphnia*, promising model organisms to investigate multiple resource limitations and to deepen our knowledge on the concept of co-limitation.

Here, using the rotifer *Brachionus calyciflorus* as a model organism, we tested our hypothesis that also rotifers can be limited by multiple nutrients simultaneously. In feeding experiments, we compared nutrient profiles (sterols, fatty acids and amino acids) of rotifers and their diets to detect consumer–diet imbalances and thus potentially limiting nutrients. In addition, we conducted population growth experiments with rotifers feeding on a nutrient-deficient diet, which was supplemented with cholesterol, the most prominent sterol in animal tissues and/or EPA, either in the absence or in the presence of an additional source of amino acids. The significance of single dietary amino acids for the performance of rotifers was assessed by experimentally increasing the availability of several putatively essential dietary amino acids.

Materials and methods

CULTIVATION OF FOOD ORGANISMS

The coccoid cyanobacterium *Synechococcus elongatus* (SAG 89-79; culture collection of the University of Göttingen) and the flagellate *Chlamydomonas reinhardtii* (SAG 11-32b) were cultured semicontinuously in modified Woods Hole Culture Medium (WC) with vitamins (Guillard 1975) at 20 °C and an illumination of 30 and 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (16-h : 8-h light/dark cycle) in aerated 1-L vessels, respectively. The molar ratio of carbon to nitrogen to phosphorus (C/N : P) of *S. elongatus* (130 : 16.5 : 1) was close to the Redfield Ratio, indicating a high nitrogen and phosphorus content of the food suspensions. Thus, a limitation by nitrogen or phosphorus was highly improbable.

The carbon concentrations of the food stock suspensions were estimated from photometric light extinctions (at 800 nm) using carbon-extinction equations determined prior to the experiment. Aliquots of algal or cyanobacterial stock suspensions were added to sterile WC medium, and the resulting suspensions of 2 mg C L⁻¹ were used as food/culture medium for rotifers. *S. elongatus* was used because it is a nontoxic, phosphorus-rich cyanobacterium that has been shown to be of poor food quality for *Daphnia* due to the lack of sterols and long-chain PUFAs (Fig. 1; Wacker & Martin-Creuzburg 2007; Martin-Creuzburg, Sperfeld & Wacker 2009).

LIPOSOME PREPARATION

The liposome stock solution was prepared from 3 mg 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and 7 mg 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC; Lipoid, Ludwigshafen, Germany) dissolved in an aliquot of ethanol. Either 3.33 mg of cholesterol or EPA (Sigma Aldrich, Deisenhofen, Germany) was added from a cholesterol or EPA solution in ethanol. Solutions were dried using a rotary evaporator and dissolved in 10 mL of buffer (15 mM [N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, TES], 150 mM NaCl, pH 7.0). Afterwards, the liposome suspension was sonicated in an ultrasonic bath for 30 s and excess free cholesterol or EPA was removed by washing the liposomes in fresh buffer using an ultra-speed centrifuge (15 000 g, 360 min,

Fig. 1. Concentrations of sterols, tetrahymanol, fatty acids, and essential amino acids in (a) *Chlamydomonas reinhardtii* and *Brachionus calyciflorus* Pallas fed *C. reinhardtii*, and (b) *Synechococcus elongatus* and *B. calyciflorus* fed *S. elongatus* (mean $\pm$ standard deviation, $n = 3$). Concentrations are given in log-scale.

4 °C). In addition, control liposomes without further ingredients were prepared. All liposome suspensions were stored at -25 °C until preparation of food treatments. The cholesterol and EPA content of liposomes was determined from subsamples; 1 μ L of the liposome suspensions contained either 0.52 ± 0.02 μ g cholesterol or 0.17 ± 0.01 μ g EPA (mean $\pm$ SD), respectively. The mean diameter of liposomes was 2.1–2.2 μ m (determined by a particle counter; CASY, Schärffesystem, Germany).

CULTIVATION OF ROTIFERS

The rotifer strain (*B. calyciflorus* Pallas) that we used in the reported experiments was originally obtained from K.-O. Rothhaupt (University of Konstanz). Since then, this strain has been used as a 'rotifer model' in numerous studies (e.g. Rothhaupt 1988; Fussmann, Weithoff & Yoshida 2005). Prior to the experiment, the rotifers were raised in glass beakers filled with 200 mL of sterile WC medium with saturating concentrations of *C. reinhardtii* (>3 mg C L $^{-1}$), to standardize experimental conditions and to start all experiments with animals in comparable nutritional state; no mixis was observed neither during preculturing nor during the experiments. Before diet changes, rotifers were repeatedly sieved using a 55- μ m mesh and rinsed with sterile culture medium to remove food organisms. To obtain sufficient biomass for chemical analyses, rotifers were reared in semicontinuous cultures in 700 mL of WC at a density of c. 10 *B. calyciflorus* per millilitre and fed either with *C. reinhardtii* or *S. elongatus*. Fresh food suspensions of *C. reinhardtii* or *S. elongatus*

were provided daily so that the food concentration in cultures was c. 2 mg C L $^{-1}$.

ROTIFER GROWTH EXPERIMENTS

To standardize experimental conditions, growth experiments were conducted with newly hatched juveniles that hatched in *S. elongatus* without supplementations. Juveniles were collected and transferred randomly to the different treatments. Experiments lasted 6 days and were conducted at 20 °C in microtitre wells filled with 10 mL food/culture suspension.

Prior to the co-limitation experiments, we conducted preliminary growth experiments using (i) WC medium without any algal diet, (ii) as before but with control liposomes, (iii) WC medium enriched with 25 mg L $^{-1}$ dissolved bovine serum albumin (BSA; Sigma-Aldrich) to provide an unspecific source of amino acids, (iv) the latter combined with liposomes and (v) *C. reinhardtii* as a reference food with a carbon concentration of 2 mg C L $^{-1}$. Without any particulate food sources such as *C. reinhardtii* or *S. elongatus*, all rotifers died within 4 days irrespective of whether they were kept on pure WC medium or on pure WC medium supplemented with BSA and/or liposomes (Fig. S1, Supporting Information). Previous tests also revealed that different concentrations of control liposomes, 0.33, 0.66, 1.3 μ L mL $^{-1}$ food suspension containing 2 mg C L $^{-1}$ *S. elongatus*, did not significantly affect growth rates of rotifers.

In the first co-limitation experiment, rotifers were fed *S. elongatus* (2 mg C L $^{-1}$) supplemented with cholesterol and/or

EPA-containing liposomes either in the absence or in the presence of BSA, that is, an additional source of amino acids. *C. reinhardtii* was used as a reference food. Liposome supplemented diets were prepared by adding 0.167 µL of liposome stock suspensions to 1 mL of food suspension resulting in 87 ng cholesterol mL⁻¹ and/or 28 ng EPA mL⁻¹ food suspension. This equals 43 µg cholesterol and 14 µg EPA mg⁻¹ cyanobacterial carbon, respectively. BSA supplemented diets were prepared by adding 25 µg dissolved BSA to 1 mL of food suspension.

In the second co-limitation experiment, rotifers were fed either unsupplemented *S. elongatus* or *S. elongatus* supplemented with one of 10 putatively essential amino acids (25 µM of food suspension). A previous test showed that amino acid concentrations of 25 µM had no detrimental effect on rotifers but were in a sufficient range to induce growth enhancing effects. Amino acids are abbreviated as follows: histidine, His; arginine, Arg; threonine, Thr; methionine, Met; valine, Val; phenylalanine, Phe; isoleucine, Ile; leucine, Leu; lysine, Lys and tryptophane, Try. *C. reinhardtii* was again used as a reference food.

The initial density of rotifers in all growth experiments was one rotifer per millilitre culture medium. At 24-h intervals, the food suspensions were completely renewed and the rotifers were counted under a stereo microscope. To avoid density dependent processes, 10 rotifers were randomly chosen and pipetted into new food suspensions of 10 mL. In case of negative population growth rates, all remaining individuals were transferred into new food suspensions. Densities of rotifers in all treatments fluctuated below those reported to cause consumer-dependent effects by direct and indirect consumer interference (Fussmann, Weithoff & Yoshida 2005). After 2 days of acclimatization, the numbers of individuals were used to calculate intrinsic growth rates for daily intervals using the equation:

$$r = \ln(N_t) - \ln(N_{t-1}) \quad \text{eqn 1}$$

where r is the intrinsic growth rate (per day), and N_t and N_{t-1} are the animal numbers on consecutive days. The population growth rate of each replicate and treatment was estimated by calculating the average value of the intrinsic growth rates of rotifers on consecutive days.

ANALYTICAL PROCEDURES

For analytical purposes, rotifers fed with *C. reinhardtii* were sieved using a 55-µm mesh and repeatedly resuspended and rinsed with sterile culture medium to remove food organisms. Then rotifers were transferred to the target food suspensions *C. reinhardtii* and *S. elongatus* (2 mg C L⁻¹). Each rotifer culture was grown for 3 days with food suspensions renewed on the second day. The cultures were sampled 1 day after the last supply of food to minimize potential contamination of the rotifer sterol, fatty acid and amino acid profiles by undigested food particles from the digestive tract. Again the rotifers were sieved, repeatedly re-suspended and rinsed with sterile culture medium. Subsamples of rotifers were counted under a stereo microscope to determine rotifer densities, and aliquots containing 7500 individuals were filtered onto a 25-mm glass-fibre filter (Whatman, GF/C; Whatman International Ltd, Maidstone, UK). Likewise, aliquots of the *C. reinhardtii* and *S. elongatus* food suspensions, corresponding to 0.5 or 1.0 mg particulate organic carbon (POC), were filtered onto Whatman GF/F filters (25 mm). Prior to extraction of sterols and fatty acids, 10 µg of 5 α -cholestan or 20 µg of tricosanoic acid methyl ester, respectively (Sigma-Aldrich), were added as internal standards. Filters were extracted with 7 mL dichloromethane/methanol (2:1; v: v) and stored under nitrogen at -25 °C until analysis. Fatty acids and sterols of diets and rotifers were analysed according to Wacker & Weithoff (2009) and Martin-Creuzburg, Sperfeld &

Wacker (2009), respectively, and normalized to the carbon content of the samples.

Prior to analysis of amino acids, the samples (stored under nitrogen at -80 °C until analysis) were hydrolysed with 6 M HCl at 110 °C for 24 h, neutralized with 6 M NaOH and analysed according to Gzik (1996). The hydrolysed and lyophilized samples were dissolved in 80% methanol and aliquots were derivatized with o-phthalaldehyde (800 mM borate buffer, pH 10.4, containing 50 mg o-phthalaldehyde, 1 mL methanol and 100 µL 3-mercaptopropionic acid) to yield stable, fluorescent derivatives. After a reaction time of 2 min, derivatives were separated by reversed-phase high-performance liquid chromatography (Knauer GmbH, Berlin, Germany) using an Aqua® column (5 µm C18 125 Å LC, 250 × 4.6 mm id; Phenomenex Inc., Torrance, CA, USA), and a stepwise gradient of acetate buffer (pH 7.0) with increasing concentration of methanol. Eluents were detected using a fluorescence detector (Model RF 551; Shimadzu, Kyoto, Japan). With this method, Try and Val as well as Gly and Thr, respectively, could not be separated. Amino acids were quantified using multipoint standard calibration curves previously determined for each amino acid and normalized to the carbon content of the sample.

For the determination of POC and nitrogen aliquots containing 1500 *B. calyciflorus* or 0.5 mg POC of *C. reinhardtii* and *S. elongatus* were filtered onto precombusted glass-fibre filters (25 mm diameter; Whatman GF/F) and after drying for at least 2 days at 50 °C analysed for carbon and nitrogen using an elemental analyser (EuroEA 3000; HEKAtech GmbH, Wegberg, Germany). For the determination of particulate organic phosphorus concentrations, aliquots of food suspensions were filtered onto polysulphone filters (25 mm, 0.45 µm; Pall Corporation, Ann Arbor, MI, USA) and analysed using the molybdate-ascorbic acid method (Murphy & Riley 1962) after dissolving tissues with sulphuric acid and oxidative hydrolysis by K₂S₂O₈ at 120 °C for 60 min.

DATA ANALYSES

Differences in population growth rates were analysed by two-way ANOVA (co-limitation experiment 1) or one-way ANOVA (co-limitation experiment 2) with subsequent post hoc comparisons (Tukey's HSD), when a factor or an interaction was significant ($P < 0.05$). Main factors were either BSA treatment and type of liposome treatment (co-limitation experiment 1) or amino acid treatment (co-limitation experiment 2). Normality was tested using the Shapiro-Wilk's test and homogeneity of variances using Flinger-Killeen's test. Additionally, statistical models were tested for linearity and constant variances. According to Crawley (2002), data of co-limitation experiment 2 were boxcox transformed with $\lambda = 3$, that is, by using $((1+y)^3 - 1)/3$ to meet assumptions. All calculations and statistical tests were conducted with the software package R, which is under general public licence (R Project for Statistical Computing, www.R-project.org).

Results

CONSUMER-DIET IMBALANCE IN NUTRIENTS

Considerable consumer-diet imbalances were detected for sterols, some n-3 PUFAs and in particular certain amino acids (Fig. 1), suggesting a dietary deficiency and thus a potential limitation of rotifer growth by these individual dietary compounds. The sterol composition of rotifers fed *C. reinhardtii* was characterized by ergosterol (IUPAC name; (22E)-ergosta-5,7,22-trien-3 β -ol), 5-dihydroergosterol [(22E)-5 α -ergosta-7,22-dien-3 β -ol], fungisterol (5 α -ergost-7-en-3 β -ol),

chondrillasterol [(22*E*)-5 α -poriferasta-7,22-dien-3 β -ol], 22-dihydrochondrillasterol (5 α -poriferast-7-en-3 β -ol), and 7-dehydroporiferasterol [(22*E*)-poriferasta-5,7,22-trien-3 β -ol]. Ergosterol and 7-dehydroporiferasterol were the principal sterols detected in *C. reinhardtii* and thus were presumably of dietary origin; the other sterols detected in the rotifers were absent from the diet. All these sterols, except 7-dehydroporiferasterol, were also detected in rotifers fed the sterol-free *S. elongatus* (Fig. 1), which suggest that these sterols were either synthesized *de novo* or originated from preexperimental feeding and were retained in the animals, (Fig. 2). This consumer–diet imbalance was evident for all sterols detected in the rotifers. In addition to sterols, the pentacyclic triterpenoid alcohol tetrahymanol was detected in the rotifers. Tetrahymanol was detected in somewhat higher concentrations in animals fed the sterol-free *S. elongatus* than in animals fed the sterol-containing *C. reinhardtii*.

The rotifers contained several long-chain PUFAs that were absent from the diets (Fig. 1). For instance, rotifers fed *C. reinhardtii* contained significant amounts of C20 PUFA that could not be detected in *C. reinhardtii*. After the diet switch to *S. elongatus*, a comparison of PUFA compositions revealed an additional consumer–diet imbalance

Fig. 2. Population growth rates (means $\pm$ standard deviations, $n = 4$) of *Brachionus calyciflorus* Pallas raised on different food regimes in a two-factorial design. One factorial treatment was the dietary presence of bovine serum albumin (BSA) in the food suspensions and the second factor was the treatment with different kinds of lipids supplemented via liposomes. Treatment with BSA has a significant effect on growth (ANOVA, $P < 0.001$), regardless of any interaction with the liposome supplementations. Since there was no interaction between both factors, bars with different letters (A or B) display significant different levels of factor two, that is, differences between lipid supplementations ($P < 0.001$; Tukey HSD following two-way ANOVA).

ance in C18 PUFAs, in particular in α -linolenic acid (ALA) and linoleic acid (LIN) (Fig. 1). Except for small differences in the concentrations of Arg and Ile, the amino acid compositions and concentrations did not differ between *C. reinhardtii* and rotifers fed *C. reinhardtii*. In contrast, a comparison of amino acid concentrations of *S. elongatus* and rotifers fed *S. elongatus* revealed a high consumer–diet imbalance in Ile, followed by a somewhat smaller imbalance in Leu, Met, Phe and Arg, suggesting a potential growth limitation by these dietary amino acids.

CO-LIMITATION EXPERIMENT 1

Population growth rates of rotifers significantly increased upon BSA supplementation by $c. 0.3 \text{ day}^{-1}$ (differences between means; Fig. 2; Table 1), irrespective of simultaneous supplementation with control liposomes or EPA-containing liposomes. Similarly, population growth rates significantly increased upon supplementation with cholesterol-containing liposomes by $c. 0.25 \text{ day}^{-1}$, irrespective of simultaneous supplementation with BSA (Fig. 2; Table 1, Tukey's HSD, $P < 0.001$ following ANOVA). Population growth rates significantly increased also by the simultaneous supplementation with cholesterol- and EPA-containing liposomes, but the obtained growth rates did not differ significantly from those obtained by cholesterol supplementation alone. The supplementation with control liposomes or EPA-containing liposomes did not significantly increase population growth rates (Tukey's HSD, $P > 0.19$).

CO-LIMITATION EXPERIMENT 2

Population growth rates of rotifers fed *S. elongatus* increased significantly upon isoleucine and leucine supplementation (Fig. 3, Table 1, Tukey's HSD, $P < 0.01$ and $P < 0.05$, respectively). All other amino acids did not significantly improve population growth rates compared to animals fed unsupplemented *S. elongatus* (Fig. 3, Tukey's HSD, $P > 0.9$). Growth rates increased upon isoleucine and leucine supplementation by 0.29 and 0.25 day^{-1} compared with unsupplemented *S. elongatus* (Fig. 3, Table 1), that is, in a similar range as observed upon BSA supplementation in co-limitation experiment 1 (Fig. 2).

Discussion

Our study shows that population growth of the rotifer *B. calyciflorus* can be limited by more than just one nutrient simultaneously, that is, by sterols and certain amino acids, which adds to growing evidence that multiple resource limitation of consumers is a common phenomenon in aquatic ecosystems. We used a controlled laboratory system with unialgal diets to control, for example, prey selection and other confounding factors potentially associated with the diversity of natural algal diets.

Table 1. Statistical analysis of the obtained population growth rates of *Brachionus calyciflorus* Pallas raised on different diets

Experiment	Factor	Population growth rate (per day)		
		d.f.	F-value	P-value
Co-limitation experiment 1	Protein treatment (BSA)	1, 30	312.3	<0.0001
	Liposome treatments (Lipo)	4, 30	26.59	<0.0001
	BSA × Lipo	4, 30	1.63	0.19
Co-limitation experiment 2	Amino acid treatment (AA)	10, 33	6.73	<0.0001

In co-limitation experiment 1, differences in population growth rates were analysed by two-way ANOVA. The factor 'liposome treatment' includes different factor levels consisting of different kinds of lipids supplemented to the diet via liposomes. The factor 'bovine serum albumin (BSA) treatment' represents the presence or absence of BSA in the food suspensions. In co-limitation experiment 2, the factor 'amino acid treatment' represents the presence or absence of single amino acids in the food suspensions (one-way design).

Fig. 3. Population growth rates (mean ± standard deviation, $n = 4$) of *Brachionus calyciflorus* Pallas raised on *Synechococcus elongatus* supplemented with one of 10 different amino acids. Bars with different letters display significant differences between amino acid supplementations ($P < 0.05$; Tukey HSD following one-way ANOVA).

Population growth rates of *B. calyciflorus* increased significantly upon cholesterol supplementation, indicating that a dietary source of sterols is required to obtain high population growth rates. As yet, our knowledge on sterol requirements of rotifers was based merely on correlative evidence. Boechat & Adrian (2006) found that egg production, but not population growth, of the rotifer *Keratella quadrata* was correlated with the dietary sterol content and concluded that a dietary source of sterols is required for rotifer reproduction. In the present study, we provide experimental evidence clearly showing that population growth rates of rotifers are constrained by a low availability of dietary sterols.

Teshima *et al.* (1981) reported that the rotifer *Brachionus plicatilis* is capable of synthesizing cholesterol, but

not C28- and C29- sterols, from radio-labelled acetate, but at the same time argued that cholesterol and the C28- and C29- sterols detected were mostly of dietary origin. In our study, we did not find any cholesterol in *B. calyciflorus* but several C28- and C29-sterols, suggesting that cholesterol is not synthesized by *B. calyciflorus* even under sterol limitation. One would expect that the putative sterol synthesising pathway is highly conserved among rotifers and that, therefore, the two species *B. plicatilis* and *B. calyciflorus* should have similar capabilities. However, although we cannot definitely infer from our data whether *B. calyciflorus* is capable of synthesising sterols *de novo*, the growth enhancing effect of sterol supplementation suggests that the animals are either incapable of synthesising sterols *de novo* or that the rates of synthesis are too low to meet physiological demands. The finding that *B. calyciflorus* maintained relatively high body sterol levels even in the absence of dietary sterols, that is, when fed unsupplemented *S. elongatus*, implies that sterols obtained from dietary sources provided prior to the experiment (i.e. from *C. reinhardtii*) were retained and partially modified within their body. However, specific time course experiments will be required to accurately assess the capability of rotifers to retain and metabolize dietary sterols. Consequently, the animals might be highly affected by changes in the sterol availability, which depends on the phytoplankton composition and environmental conditions (Piepho, Martin-Creuzburg & Wacker 2010).

In addition to sterols, the pentacyclic triterpenoid alcohol tetrahymanol was detected in the rotifers, although it was absent from the diet, suggesting that rotifers are capable of synthesising tetrahymanol *de novo*. In ciliates, tetrahymanol is produced as sterol surrogate in the absence of dietary sterols and has been shown to be functionally equivalent to sterols as a structural component of cell membranes (Ourisson, Rohmer & Poralla 1987; Harvey & McManus 1991; Martin-Creuzburg, Bec & Von Elert 2006). To our knowledge, this is the first time that tetrahymanol has been detected in a rotifer. The tetrahymanol content of rotifers was not significantly affected by the availability of dietary sterols, suggesting that tetrahymanol cannot serve as sterol surrogate in rotifers, but this should be subjected to further investigations.

Supplementation with EPA did not affect population growth rates of *B. calyciflorus* (Fig. 2), suggesting that a dietary source of EPA is not required. However, this is in contrast to previous studies reporting that egg production of the rotifer *Keratella quadrata* fed ciliates was correlated with the availability of dietary PUFAs, in particular EPA (Boechat & Adrian 2006). Recently it has been shown that environmentally induced changes in dietary PUFA availabilities (Spijkerman & Wacker 2011) strongly affect the growth of rotifer species from acidic mining lakes (Weithoff & Wacker 2007) and their competitive abilities (Hartwich, Wacker & Weithoff 2010). These contradictory findings suggest that different dietary constraints arise from feeding on cyanobacteria, as in the present study, and eukaryotic food sources, as in the cited studies.

The ecological relevance of a dietary deficiency in essential amino acids is potentially high, as suggested by large consumer–diet imbalances in certain amino acids found in several aquatic and terrestrial consumers (Anderson, Boersma & Raubenheimer 2004). In general, the amino acid content of invertebrate (Øie & Olsen 1997; Tidwell *et al.* 1998; Helland *et al.* 2003) and vertebrate (Alam *et al.* 2002) consumers tends to be regulated homeostatically, that is, despite considerable changes in dietary amino acid availabilities, changes in the amino acid content of consumers are rather low. The tendency of consumers to maintain certain amino acid concentrations within their body may increase the risk of a limitation by single amino acids, in particular by those amino acids which are temporarily underrepresented in the diet (Kalachova *et al.* 2004). This assumption is supported by the observation that the hatching success of copepods is improved when differences in the amino acid composition between females and their diet are less pronounced (Guisande, Maneiro & Riveiro 1999; Guisande, Riveiro & Maneiro 2000; Helland *et al.* 2003). In the theoretical approach of Anderson, Boersma & Raubenheimer (2004), a comparison of amino acid profiles of copepods and their phytoplankton prey revealed a significant consumer – diet imbalance for arginine and threonine, suggesting that the performance of copepods is potentially constrained by the dietary availability of these amino acids. Following the same approach, our data revealed considerable differences in the concentrations of certain amino acids, in particular leucine and isoleucine, between *S. elongatus* and rotifers fed *S. elongatus*, suggesting a potential growth limitation of rotifers by leucine and isoleucine (Fig. 1). This was corroborated by the amino acid supplementation experiment, clearly showing that the population growth of *B. calyciflorus* on *S. elongatus* was in fact constrained by the availability of leucine and isoleucine.

The hypothesis that isoleucine is of general importance for invertebrate growth and reproduction is supported by the finding that a lack of isoleucine in the diet of the Mediterranean fruit fly, *Ceratitis capitata*, resulted in lowest larval growth compared to the absence of other potentially essential amino acids, except for threonine and tryptophan

where no larvae survived (Chang 2004). The beneficial effect of leucine on population growth of *B. calyciflorus* is in accordance with a previous study showing that egg production of the rotifer *Keratella quadrata* was correlated, among other biochemical nutrients (fatty acids and sterols), with the leucine content of its protist food source (Boechat & Adrian 2006).

The availability of amino acids might correlate well with that of nitrogen, and nitrogen was suggested as surrogate measurement for amino acids (Jensen *et al.* 2006; Hessen *et al.* 2007). Therefore, one might speculate that the observed positive effects of BSA or amino acid supplementation are in fact due to the increased availability of nitrogen rather than to the addition of essential amino acids. However, in our direct supplementation approach we did not find evidence for a potential limitation of the rotifers by particularly nitrogen-rich amino acids, such as arginine or histidine. Only the addition of leucine and isoleucine (with low nitrogen contents) improved population growth rates of *B. calyciflorus* (Fig. 3), clearly indicating that the rotifers were limited by the low dietary availability of these essential biochemical molecules and not by the availability of dietary nitrogen. In general, the dietary supply of elemental nutrients was high in the present study and thus a limitation of *B. calyciflorus* by phosphorus or nitrogen appears unlikely. Moreover, the supplementation of *S. elongatus* with control liposomes, which provide an extra source of carbon and phosphorus (Martin-Creuzburg, Von Elert & Hoffmann 2008), did not improve population growth rates, showing that phosphorus supply and food concentrations in general were sufficient. Hence, studies investigating nutritional requirements of rotifers should also consider essential biochemicals as potentially limiting nutrients and should not merely focus on insufficient dietary supplies of minerals such as phosphorus and nitrogen as has been done in the past (Rothhaupt 1995; Strojsova *et al.* 2010).

We show here, for the first time, that the rotifer *B. calyciflorus* relies on a dietary source of sterols and that a dietary sterol-deficiency results in reduced population growth rates, as indicated by a growth enhancing effect of sterol supplementation. Similarly, we show that population growth rates of *B. calyciflorus*, on the same nutrient-deficient diet, can be improved by amino acid (leucine, isoleucine) supplementation, indicating a simultaneous limitation by sterols and amino acids. The substantial increase in growth rates as a response to sterol and amino acid supplementation demonstrates that a dietary deficiency and not the production of toxins or growth inhibitors are responsible for the low food quality of *S. elongatus*. Rotifers significantly feed on cyanobacteria, even if these are toxic (Snell 1980; Walz 1995; Soares, Lurling & Huszar 2010). Hence, our data suggest that the growth of rotifers is potentially constrained simultaneously by a deficiency in dietary sterols and certain amino acids. This might be particularly relevant when the phytoplankton is dominated by cyanobacteria. Although the addition

of these biochemical compounds considerably improved the food quality of *S. elongatus* for *B. calyciflorus*, population growth rates obtained on the supplemented diets were significantly lower than those obtained on a sole diet of *Chlamydomonas* (Figs 2 and 3), suggesting that other food quality constraints become important once the sterol and amino acid requirements are met.

Although our approach is straightforward in identifying limiting dietary compounds it does not consider the possibility of using complementary food sources to obtain nutrient balanced diets or compensatory feeding, that is, the increased uptake of certain food sources to satisfy the requirements for single dietary nutrients (Raubenheimer & Jones 2006). However, during extensive cyanobacteria blooms complementary and compensatory feeding might be of minor importance because rotifers potentially do not have sufficient alternative food sources and may depend on potential improvement of the sterol and amino acid availability through seasonal changes of the phytoplankton community.

We show here, for the first time, that an aquatic invertebrate is simultaneously constrained (co-limited) by a deficiency in dietary sterols and certain amino acids. Previous work using the same food organism reported a similar scenario for a completely distinct phylogenetic group of aquatic invertebrates (Crustaceae, *Daphnia*), which however was co-limited by sterols and fatty acids instead of amino acids. This suggests that (i) co-limitation among consumers is more widespread as expected, and that (ii) biochemical nutrient requirements differ among phylogenetic groups. A limitation of rotifers by these essential biochemical nutrients may also affect the functioning of freshwater food webs, as rotifers are significantly involved in the carbon transfer to higher trophic levels. Rotifers were successfully used to study mechanistic resource competition theory (Rothhaupt 1988) and for aquaculture purposes (Hagiwara *et al.* 1993; Makridis & Olsen 1999; Aragao *et al.* 2004; Srivastava *et al.* 2006). The present study shows that they may also be of high value to study and characterize resource co-limitation scenarios.

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