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RNA responses to N- and P-limitation; reciprocal regulation of stoichiometry and growth rate in *Brachionus*

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Summary

1. In this study we address how growth rate in consumers may be regulated by nitrogen (N)- and phosphorus (P)- limitation of ribonucleic acid (RNA), using the rotifer *Brachionus calyciflorus* as the model organism.

2. Growth rate, RNA and weight-specific concentrations of carbon (C), N and P were analyzed in *B. calyciflorus* fed algae with different C : N, C : P and N : P ratios.

3. Growth rate correlated negatively with food C : N, but not with C : P or N : P, strongly suggesting N-limited growth. Growth rate also strongly correlated with tissue concentrations of RNA and P, in support of the growth rate hypothesis which states that specific P-content of organisms reflects their RNA-content and thus capacity for protein synthesis.

4. Food C : N rather than C : P regulated the growth, RNA and P in this consumer under the food conditions applied. This suggests that availability of N can also be an important regulator of cellular acquisition of P and build-up of ribosomes in that animals released from N-limitation have an increased demand for the P-rich cellular machinery for protein synthesis.

5. The data suggest a strong reciprocal regulation of consumer demands and their C : N : P stoichiometry via the intimate connections between ribosome allocation and protein synthesis and also lend support to the view that reduced protein synthesis efficiency under high growth rates may affect stoichiometry of the consumer.

Key-words: *Brachionus*, growth rate, N-limitation, RNA, stoichiometry

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Introduction

What are the resource constraints for growth in organisms? The commonly held view has been that autotrophs are limited by their access to nutrients, solar energy and water; consumers are primarily limited by their access to energy. More recently, there has been increased focus on potential food 'quality' limitation in consumers, due to insufficient dietary supplies of N (amino acids, proteins) and P (Hessen 1992; White 1993; Sterner & Elser 2002). Both N and P are crucial for protein synthesis and thus growth rate, and because herbivorous consumers generally have higher mass-specific N- and P-contents than that of their plant or algal food, their growth may be rate limited by a shortage of these elements.

While the role of dietary protein deficiency (and thus implicit N-limitation) has been strongly advocated for many organisms (cf. White 1993), the role of P as potentially limiting for ribonucleic acid (RNA) has been proposed in other studies. Since ribosomes are the site of protein synthesis, this means that P-deficiency could constrain protein synthesis and thus growth. Based on early simple mass-balance models, it was assumed that P-rich herbivores such as the crustacean zooplankter *Daphnia* were likely candidates for P-limited growth rates, and this has repeatedly been experimentally verified (see Sterner & Elser 2002 for review). For many P-rich and fast-growing invertebrates, RNA constitutes a major fraction of body P, suggesting a close association between the specific P-content and growth rate of an organism (Hessen 1990; Andersen & Hessen 1991; Sterner & Hessen 1994; Elser *et al.* 1996). Thus, in terms of classical life-history theory, it can be proposed that among the studied crustaceans, specific P-content reflects their life-history strategy along an r-K continuum. However, such comparisons may not be valid across different phyla, since body size and complexity undoubtedly play a major role. For example, freshwater members of the Phylum

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Rotifera (rotifers) generally possess far higher growth rate than crustaceans, yet the limited stoichiometric data available indicate that they do not have higher P-content than crustaceans and seem more frequently limited by N than P in their diet (Rothhaupt 1995; Jensen & Verschoor 2004).

Elser *et al.* (1996) formalized the link between cellular P and organismal growth as the Growth Rate Hypothesis (GRH), suggesting that high biomass P-content reflects increased allocation to P-rich ribosomal RNA needed to meet the protein synthesis demands of increased growth rates. These assumptions seem to be met for a wide range of prokaryotes and arthropods (Vrede, Persson & Aronsen 2002; Elser *et al.* 2003; Acharya, Kyle & Elser 2004a,b; Weider *et al.* 2005; Karpinets *et al.* 2006). Thus, any process of direct or indirect natural selection affecting growth and development capabilities, or physiological adjustment leading to changes in growth rate, could contribute to differences in the C : N : P ratios of the biomass being formed by a given organism.

The biochemical interpretation is generally that, by increasing P supply, the cellular ribosome copy number increases up to a certain level where translational capacity is 'saturated' and the growth rate eventually levels off due to either N (or amino acid) limitation or C (energy) limitation. Despite the success of the GRH, it is important to recognize that P, N and C play multiple roles both in catabolic and anabolic pathways. P is also important as a carrier of energy (AMP, ADP, ATP) as well as a component of membrane phospholipids, and both proteins and carbohydrates serve both as building blocks and energy carriers. It is also clear that under strong N-deficiency, ribosome synthesis could also be N-limited since proteins are N-rich (*c.* 15% N of DW) and ribosomal proteins have a significant allocation at high growth rate. Hence more P for RNA would not be expected to enhance growth under these circumstances.

Thus, the causal basis for a close association between growth rate and P is not straightforward. For consumers whose growth rate is primarily limited by the P-content in their diet (like the many examples of *Daphnia* feeding of high C : P diets, cf. Sterner & Elser 2002), the causal direction is no doubt from P to RNA to growth. Under N-limitation, however, the coupling between P, RNA and growth rate may be relaxed (Elser *et al.* 2003). For organisms limited by other minerals or essential food quality components (like essential vitamins, sterols, fatty acids or amino acids), increased access to these may allow for increased growth, thereby inducing a secondary demand for RNA and thus for P. Thus under N-limitation, increased N supply would allow for increased protein synthesis, demanding increased ribosome synthesis which would be reflected by increased specific content of RNA or P. Of course, other organisms could experience the reverse causality depending on their nutritional status and N : P ratio in food relative to their demands.

We thus propose that for organisms that primarily face dietary protein (or N) limitation, increased cellular concentrations of RNA could only occur when the consumer is relaxed from N-limitation. This means that cellular P-content will be regulated by the access to N, rather than P. Here we use data on growth rate, body RNA content, and C : N : P stoichiometry in the rotifer *Brachionus calyciflorus* to test how these parameters in this consumer are influenced by food (algal) stoichiometry. The reason for choosing this species is that is a likely candidate herbivore for N-limitation (Rothhaupt 1995; Jensen & Verschoor 2004), it is fast-growing, asexual (clonal) and ideal for this kind of growth assays and that it could serve as a model organism for the reciprocal regulation of N and P-limitation in consumers.

Materials and methods

The experimental protocols follow Jensen & Verschoor (2004) and Jensen *et al.* (2006). The data were obtained from samples generated during the experiments of Jensen *et al.* (2006) plus additional analyses. In brief, the green alga *Selenastrum capricornutum* was used as the sole food during these experiments. The algae were grown in continuous cultures (dilution rate 0·2 day^{-1}) in 2 L polycarbonate vessels (Nalge Company) equipped with magnetic stirrers. The cultures received 70 µmol quanta $m^{-2} s^{-1}$ PAR-light from 25 W blue–white fluorescent tubes. Cultures were allowed to run for 2 weeks before start of the experiments in order to obtain stable cultures. The algae were cultured on the same three types of COMBO medium (Kilham *et al.* 1998) used by Jensen & Verschoor (2004): full nutrient-sufficient medium (F), P-depleted medium (–P), and N-depleted medium (–N) with four replicates of each treatment. This yielded a consistent difference in algal C : N and C : P ratios among the three treatments. For the feeding experiments we pooled the algae from the four chemostats within each treatment.

The same food concentration (6 mg C L^{-1}) was used across all experiments. The food was replaced daily. As a proxy of cell density, daily measurements of optical density at 663 nm (UV-210A, Shimadzu Seisakusho Ltd) was used based on a standardized conversion from absorbance and algal C. Twice a week, samples for determination of phytoplankton size distribution and particulate C, N and P were taken. Samples for size distribution of the algae were fixed with Lugol (1% final concentration). Cell number was determined by visual microscopic inspection while cell size distribution was determined by flow cytometer (CASY TTC1). Subsamples from the cultures were filtered on pre-combusted, acid washed GF/F filters for analysis of particulate C, N and P. C and N were analyzed on a Flash EA™ 1112 automatic elemental analyzer (Thermo Finnigan, Milan, Italy). Particulate P samples were analyzed by colorimetric analysis following oxidation according to Hessen, Færøvig & Andersen (2002).

Algae from the different cultures were used as food in the zooplankton experiments. Harvested algae were standardized to the desired food concentration by diluting with nutrient-free COMBO medium. This concentration was calculated from previously established regressions for each food type between absorbance and carbon content of the cultures.

Brachionus calyciflorus was hatched from dormant eggs (MicroBioTest inc., Nazareth, Belgium). This insured identical egg quality and synchronized age of the population. Rotifers were cultured in COMBO medium without phosphate and nitrate to avoid uptake of N and P from the medium by the food algae. Ionic strength of the nutrient-free zooplankton medium was maintained by the addition of KCl in a concentration of 100 μmol L^{-1}. All experiments were carried out at constant temperature (19 °C) in a thermostatically controlled room under dim light. *Brachionus calyciflorus* for the experiments were hatched from cysts placed in nutrient-free medium in shallow dishes. Hatching started after 36 h of incubation at 19 °C.

The stoichiometric composition of *B. calyciflorus* fed algae differing in quality was measured. For each food quality, four replicates with 300 individuals in each were transferred to small acid washed glass vials in 10 mL of distilled water and then filtered onto pre-combusted acid washed GF/F filters before being analyzed for C, N and P as described above. From the same rotifer cultures samples were also taken for determination of dry weights. For this purpose a known number (70–100) of rotifers were transferred to pre-weighed silver cups, dried for 24 h at 60 °C, cooled in a desiccator and weighed to the nearest μg on a microbalance (Mettler Toledo MX5). *Brachionus calyciflorus* fed the different algae qualities were also analyzed for RNA content following Kyle *et al.* (2003). After washing animals twice in distilled water, individuals were transferred with a narrow Pasteur-pipette to Eppendorf vials containing 100 μL distilled water and 900 μL RNA-Later™ was added to the sample (*c.* 100 individuals per sample). The samples were stored for 24 h in a refrigerator, and then kept at –20 °C until analysis. To ensure complete quantification of the RNA after preservation in RNA-Later™, it was necessary to remove the rotifers from the solution prior to extraction. Therefore, *c.* 10–12 rotifers per sample were removed from the RNA-Later™ using a 10-uL pipette and then transferred to a microcentrifuge tube containing *N*-lauroylsarcosine (Sigma) buffer for extraction. The extraction was aided by sonification using a cup horn and the nucleic acid was then stained with RiboGreen (Molecular Probes) and fluorescence read using a plate fluorometer (Bio-Tek FLx800) following methods described in Kyle *et al.* (2003). RNA-content was analyzed in five replicate batches of animals for each treatment.

The somatic growth of juvenile *B. calyciflorus* was measured for each food quality. These growth experiments were conducted according to Jensen & Verschoor (2004), except that animals were offered food concentration of 6 mg C L^{-1} during the whole experimental period of 24 h. In brief, individual growth for 24 h was assessed microscopically on 35 individuals for each food treatment.

The effects of the food quality treatments were assessed by one-way ANOVA, followed by a Tukey–Kramer HSD test for comparison of specific treatments. Least square linear regression analysis was used to test for associations among parameters. All statistical analyses were performed with JMP 5·01 (SAS Institute Inc., Cary, NC).

Results

The manipulation with nutrients and light yielded three distinctively different food qualities (Fig. 1). Full

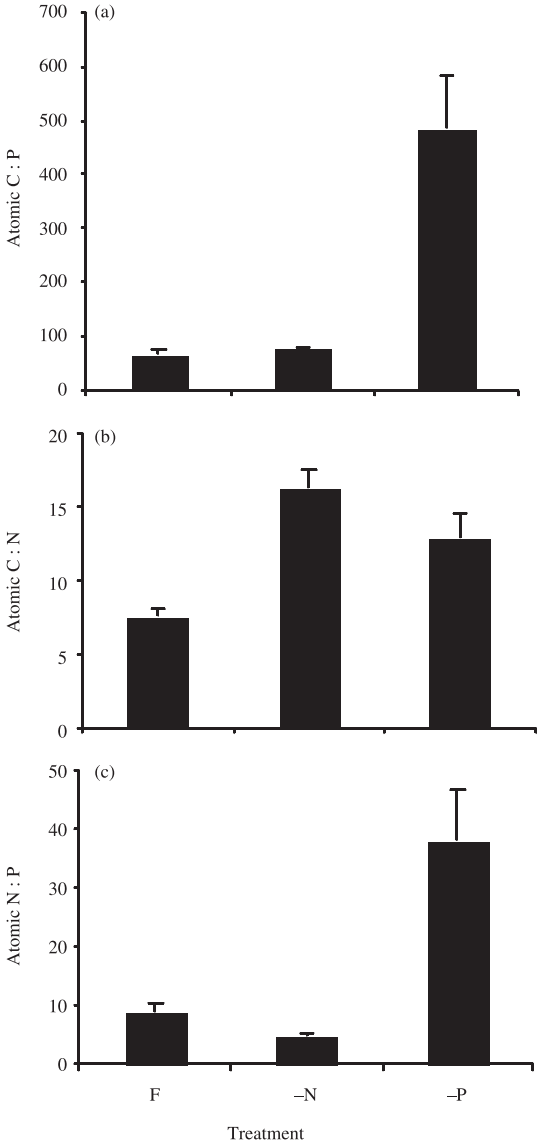

Fig. 1. Atomic C : P, C : N and N : P-ratios in phytoplankton (the chlorophyte *Selenastrum*), used as food in the various treatments. Mean of four replicate cultures with standard deviation as vertical bars.

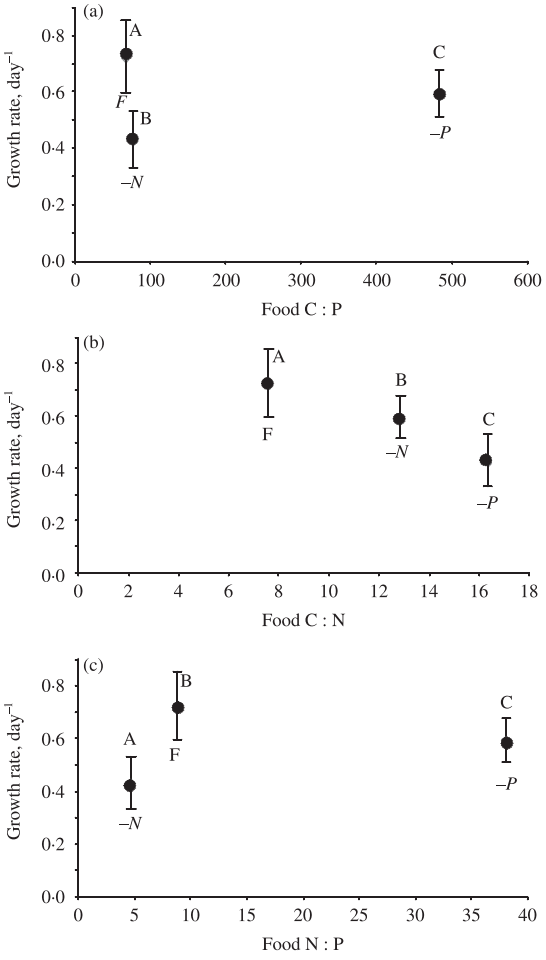

Fig. 2. Rotifer growth rate over C : N, C : P and N : P-ratios in their food. Means of 35 individuals and standard deviation with letters on top (A, B, C) denotes significant differences when different letters (Tukey HSD test). Treatments are denoted below symbols.

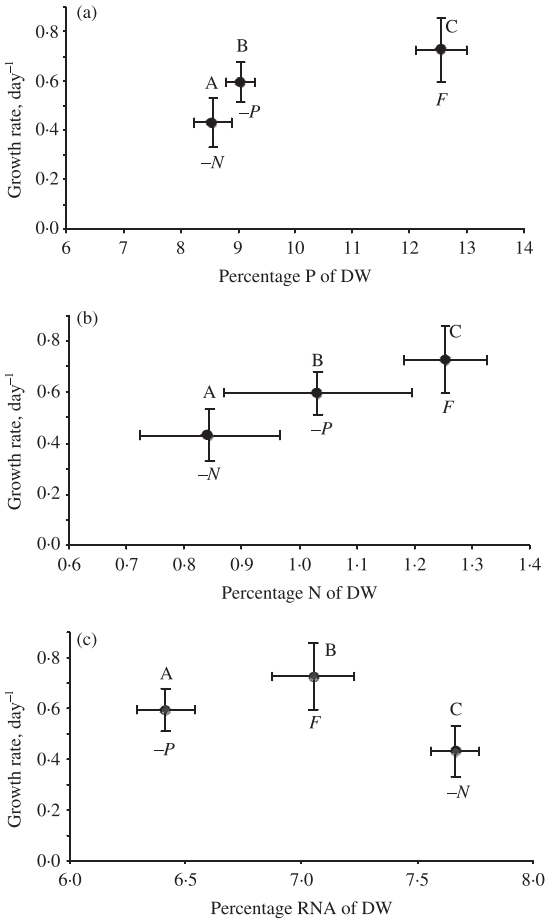

Fig. 3. Scatterplots of growth rate over specific P-content and RNA in the consumer. Mean values and standard deviations of four replicate measurements of RNA for each food type. Legends as in Fig. 2.

food (F) had both low C : P and C : N ratios, P-limited food (–P) had highly elevated C : P-ratios (484), while N-limited algae (–N) had the highest C : N-ratios. It is noteworthy however, that –P food also had somewhat elevated C : N-ratios (as observed in other experiments) while there was a less pronounced effect of the –N treatment on algal C : P-ratios. Hence, the –P treatment produced algae depleted both in terms of N and P.

Rotifer growth rate decreased with increasing algal C : N ratio ($P < 0{\cdot}001$ by least square linear regression) but there was no obvious trend of growth rate with food C : P or N : P, despite significant differences in growth among the three treatments (Fig. 2a–c). There was also a strong, positive correlation between specific P-content and RNA in the consumer. Assuming a P-content in RNA of *c.* 9% (Sterner & Elser 2002), RNA-P on average contributed 87% ± 0·08% (mean ± 1 SD) of total P. This percentage differed significantly among food treatments (90% ± 6% (mean ± 1 SD), 79% ± 4% and 91% ± 7% for animals fed F, –P and –N algae, respectively; one-way ANOVA, $F_2 = 5{\cdot}16$, $P = 0{\cdot}03$). Pair-wise comparisons showed that animals fed –P algae had a significantly lower percentage of P in RNA than individuals fed –N algae (Tukey's HSD test, $P < 0{\cdot}05$). Consumer growth rate had a significant positive correlation ($P < 0{\cdot}001$) with both specific P- and RNA-content (Fig. 3a,b), while specific N-content of the consumer did not correlate with growth rate (Fig. 3c). The high specific N-content of *Brachionus* in the –P treatment is notable.

Body specific P-content differed between the F and –N, treatments but not between the –P-treatment and the others in pair-wise testing (*t*-test, $P < 0{\cdot}05$) (Fig. 4). Pair-wise comparisons did not yield significant differences between treatments, however, neither in total (one-way ANOVA, $F_2 = 5{\cdot}17$, $P = 0{\cdot}026$), nor with Tukey's HSD test ($P > 0{\cdot}05$ for all pairs). Nevertheless the average P-content of animals in the F treatment was 49% higher than for animals having the lowest growth rates from the –N treatment. This lack of significance likely reflects the large variation in the data. Despite the lack of significance of food treatment effects on body P content, the negative correlation between food C : N and consumer P content was highly significant ($P < 0{\cdot}001$).

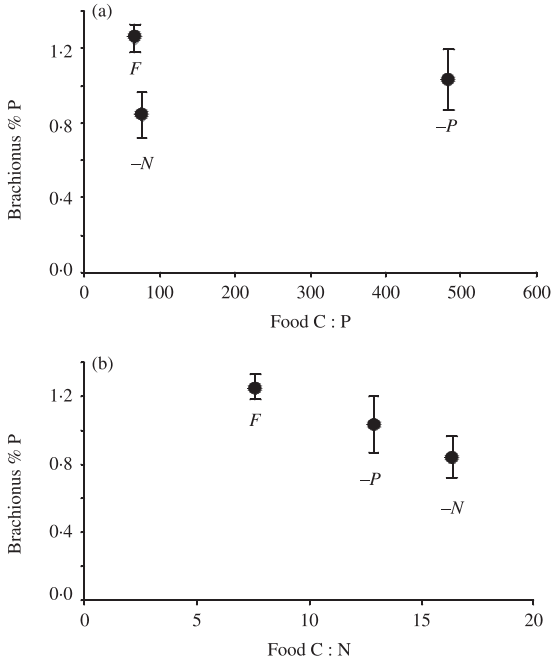

Fig. 4. P (as % of dry weight) in the consumer over C : N or C : P content of the food. Mean values and standard deviations of four replicate measurements for each food type.

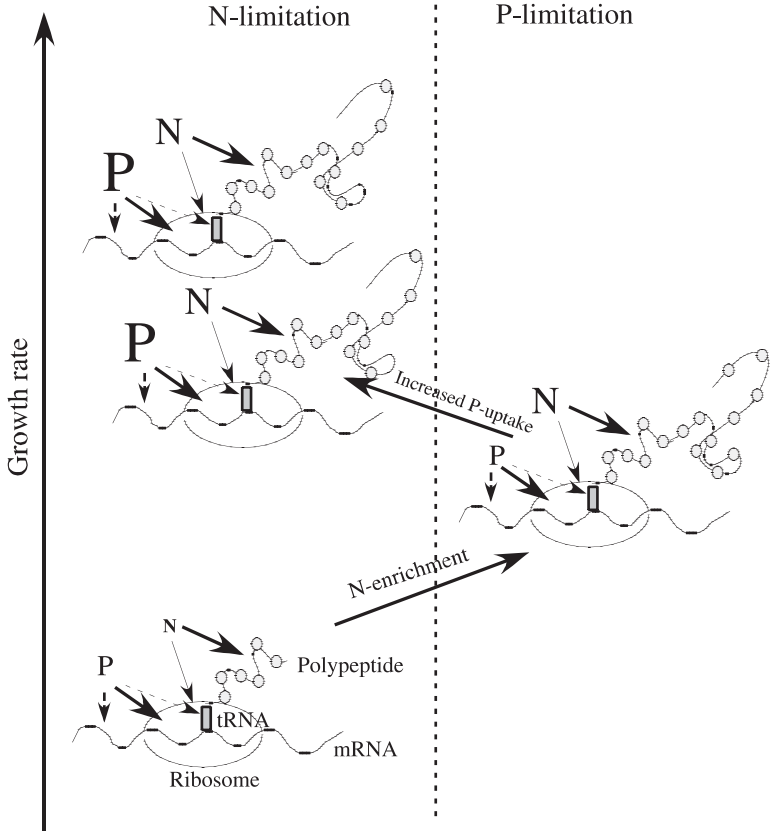

Fig. 5. Conceptual graph illustrating a simplified scenario for the reciprocal regulation on N and P-limitation via ribosome and protein synthesis. In this case consumer growth rate is limited by N as building blocks for amino acids, hence the peptide elongation is constrained. By enriching food with N the consumer is released from its primary limitation and peptide elongation speeds up, inducing temporary P-limitation and increased demand for P. This could activate both rDNA genes and genes regulating phosphatase synthesis. If there is sufficient P in food this could yield increased ribosome numbers, increased overall protein synthesis and thus growth rate.

Discussion

Our data suggest that *Brachionus* growth was limited primarily by the availability of N (as a proxy of amino acids or peptides) in food in these experiments (Jensen *et al.* 2006). Rothhaupt (1995) demonstrated reduced growth rate in *Brachionus* on nutrient-limited algae and also argued that this was due to N-limitation and not by reduced ingestion rates of nutrient-deficient food.

C : N-ratios in food correlated negatively with the consumer's P- and RNA-contents, but food C : P did not. Nitrogen thus appeared to be the primary driver for P-content, RNA-content and growth rate in *Brachionus*. One interpretation of these associations is that increased N in food stimulates protein synthesis, inducing an increased cellular demand for rRNA to support elevated translation and thus also increasing P-content. This leads to the somewhat counterintuitive observation that rotifers had higher P- and RNA-contents under extreme P-limitation (–P treatment) than under N-limitation (–N treatment). This seems to be mirrored by the response in the algal cells where especially the P-limited treatment yielded not only elevated C : P-ratios but also elevated C : N-ratios. Even with P-deficient diet, RNA made up close to 80% of total P, meaning that RNA : DNA-ratio must have been > 8, assuming that some smaller but relatively fixed fractions of P were allocated to ATP and phospholipids (Sterner & Elser 2002). This extremely high P-investment in ribosomes appears to reflect a life cycle strategy geared for high growth rate (Elser *et al.* 1996).

One puzzling outcome of these experiments was the observation of low N-content in *Brachionus* with intermediate growth rate in individuals fed –P algae. That is, high specific N-content in food yielded high P- and RNA-contents and growth rates but not high body N-content. Unlike the situation for P, however, only a modest fraction of total body N is likely to be directly involved in biosynthesis (i.e. in ribosomes themselves). Most N will likely be allocated to structural proteins, and thus one should not expect the same strong link between body N and growth as with P.

Here we propose a conceptual model (Fig. 5) to explain the observed associations. When consumers are released from their proximate limiting element or substance (for *Brachionus* in these experiments apparently N or amino acids), growth will increase until eventually limitation is shifted to other substances or elements (such as P) in line with the Liebig's minimum principle (stating that growth in organisms is not controlled primarily by the total amount of resources available, but by the scarcest nutrient or resource). However, since N and P are so closely associated with the biosynthetic machinery of growth itself, limitation by these key resources may shift predictably along growth rate gradients. Specifically, growth rate response along gradients of N and P supply may depend on the relative

allocation to various biochemical compounds during growth. A first attempt to explore this in a stoichiometrically explicit model was made by Dobberfuhl and Elser (Dobberfuhl & Elser 2000; Sterner & Elser 2002), where N : P in the consumer is expressed as a function of growth rate. The model predicts that % N increases slightly with growth rate, but over the full range of variability in protein content (30%–70% of body mass) and RNA (1%–20% of body mass), specific N-content varies only by a factor of 2. The increase in P is much faster with increased growth. Hence, with increased maximum growth rate the N : P ratio will converge towards 7, the N : P ratio of a ribosome itself. The fact that the highest specific N content (7·7% of DW) for *Brachionus* was found for the lowest specific growth rate does not necessarily conflict with the Dobberfuhl and Elser model; it could simply reflect another allocation scheme under strong N-deficiency.

The relationship between ribosomes and protein synthesis is also a matter of protein synthesis efficiency (the fraction of synthesized protein that is retained in new biomass). Karpinets *et al.* (2006) demonstrated a strong negative correlation between N : P ratio and growth rate in a number of bacteria, strongly supporting the GRH. The same pattern was seen in *Brachionus*, where N : P ratio also decreased with growth rate. Karpinets *et al.* also found a slope > 1 for the correlation between the cellular RNA : protein ratio and the number of active ribosomes per synthesized protein, suggesting that the protein production efficiency decreases with increased growth rate. This likely reflects a balance between resources allocated to growth and those required for maintenance. For prokaryotes and lower eukaryotes, the RNA : protein (or P : N) ratio may thus be a consequence not only of growth rate-related biochemical allocations, but also the effect of growth rate on the overall efficiency of protein production (Karpinets *et al.* 2006). In our study there was a tight positive correlation between growth rate (GR) and the RNA : N-ratio (RNA : N = 2·23GR + 0·13, r^2 = 0·98). Assuming that N is a proxy of protein, the slope suggests a increase in RNA : N and hence an decreased protein synthesis efficiency with increased growth rate.

In conclusion, our data provide strong support for the GRH by showing close positive associations among growth rate and RNA- and P-contents. However, the apparently N-limited *Brachionus* we studied gave a somewhat different stoichiometric response than demonstrated for commonly P-limited consumers like *Daphnia*. Whether these associations exist for other kinds of food limitation remain to be seen. For example, Jensen & Verschoor (2004) proposed polyunsaturated fatty acids as possible constraints on growth. These may play in concert with limitation by elements or other macromolecules and obscure the correlations between growth rate and food stoichiometry. Nevertheless, whatever constituent is deficient in food, the ribosomal machinery regulating body growth is tightly linked with P. In our experiments with *Brachionus*, the availability of N was a more important regulator of cellular allocation to RNA and thus of body P content even in the –P treatment, indicating that, when released from N-limitation, there is a generalized increased demand for the cellular machinery for protein synthesis. Our study suggests a strong reciprocal regulation of consumer growth rate, biochemical composition, and C : N : P stoichiometry via the balance of ribosomal and protein synthesis. Our data also suggest that reduced protein synthesis efficiency under high growth rates may affect cellular stoichiometry of the consumer.

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