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Biological stoichiometry of *Daphnia* growth: An ecophysiological test of the growth rate hypothesis

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Abstract

The growth rate hypothesis (GRH) proposes that variation in organism C : P and N : P ratios reflects variation in P content associated with altered allocation to P-rich ribosomal RNA under different growth rates. We tested the GRH by examining the effects of food quantity and stoichiometric quality (differing carbon : nitrogen : phosphorus [C : N : P] ratios) on juvenile growth and chemical composition (C : N : P, RNA, and DNA contents) of two species of *Daphnia* (*D. galeata*, *D. pulicaria*). *Daphnia* in P-limited experiments were fed saturating and limiting concentrations of food (*Scenedesmus acutus*) of high P (C : P = 110 ± 7.3), medium P (456 ± 20.7), and low P (934 ± 23.6), and in an N-limited experiment *D. pulicaria* was fed saturating concentrations of high N (C : N = 6.31 ± 0.35), medium 1 and medium 2 (9.0 ± 0.42; 15.0 ± 0.49, respectively), and low N (18.22 ± 0.56) food. In P-limited experiments, both *Daphnia* species grew fastest under P-rich, high food conditions and grew slowest under P-deficient, low food conditions, showing effects of both food quality and quantity. *Daphnia* body percentage P, C : P, N : P, and percentage RNA were tightly correlated with growth rates, and RNA contributed a significant fraction of total body P (48.8% [±2.0%]). This strong three-way (growth–RNA–P) set of correlations supports the GRH. In the N-limited experiment, food C : N had a moderate effect on *Daphnia* growth. While there was a good linear correlation between P and RNA, growth rate was uncorrelated with RNA content and P content, suggesting that the three-way coupling of growth, RNA, and P content is broken under N limitation of growth, but more data for these conditions are needed. These data help in delineating the physiological conditions under which the GRH holds and may be useful in interpreting variation in body stoichiometry of zooplankton from field and lab studies.

Growth rate is a critical parameter for most animals, since it affects age at first reproduction with consequences for reproductive output, adult body size, predation risk, and other key aspects of a species' life history (Arendt 1997). Indeed, for the crustacean zooplankter *Daphnia*, instantaneous somatic growth rate of juveniles varies in the same way as intrinsic rate of population increase (Lampert and Trubetskova 1996) and can be used as a direct correlate of overall fitness (McCauley et al. 1990). This close association occurs because *Daphnia* allocates a fixed proportion of its total production to reproduction regardless of variations in absolute growth rate due to environmental conditions. While population increase has often been used as a measure of fitness of genotypes under certain environmental conditions (e.g., Weider 1993), field and laboratory measurements of population demography are time consuming and laborious. Thus, development of approaches for reliable estimation of individual growth rates would represent a major step forward in the study of the ecology of *Daphnia* and allow a better understanding of how *Daphnia* growth responds to a variety of important environmental factors.

Growth of *Daphnia* is generally determined by the availability and quality of food, in addition to several abiotic factors (such as temperature and pH). Phosphorus (P) is one important aspect of food quality for freshwater zooplankton (Gulati and DeMott 1997) and has been the focus of considerable recent study (Sterner and Elser 2002). In lab and field studies, *Daphnia* growth has been shown to correlate well with algal P content when food P content is below threshold levels (Urabe and Watanabe 1992; Sterner et al. 1993; Gulati and DeMott 1997). P supplementation experiments have shown that at least some of this growth reduction is a direct result of dietary P deficiency (DeMott et al. 1998; Boersma and Kreutzer 2002; Sterner and Elser 2002). In contrast with P, the role of nitrogen in determining food quality has received considerably less attention in nutritional studies of freshwater zooplankton. This is partly because production in aquatic systems generally is thought more likely to be limited by P (but *see* Elser et al. 1990) as opposed to terrestrial systems, which are thought to be mostly N limited. However, White (1993) argues that the growth of many animals is constrained more by dietary protein (N) supply than by energy intake. In one study of the effects of N nutrition on a freshwater zooplankter, Sterner et al. (1993) fed *Daphnia obtusa* N- or P-limited algae (with high C : N and C : P ratios, respectively) and found that *Daphnia* fed P-limited food performed more poorly compared to animals receiving N-limited food with higher P content. Thus, *Daphnia* growth was more consistently predicted by the P content of the food than by protein or N (Sterner and Elser 2002). In contrast, effects of dietary N status on marine crustacean zooplankton have been documented (Kiørboe 1989). Given that phytoplankton N limitation may be more prevalent in freshwaters than is generally acknowledged (Elser et al. 1990), N deficiency may also play a role in freshwater food webs and needs more examination.

The framework of ecological stoichiometry (Sterner and

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Elser 2002) provides means for understanding the effects of nutritionally unbalanced foods on herbivorous animals, for establishing the mechanistic basis of impacts of dietary nutrients on animal performance, and for solving the practical problem of assessing growth rate under various food conditions. These means arise via mechanisms articulated in the growth rate hypothesis (GRH), which argues that variation in the P content (percentage of body dry weight) of animals reflects altered allocation to P-rich RNA associated with differences in growth rate, since increased growth requires elevated allocation to RNA-rich ribosomes. Thus, foods become nutritionally unbalanced in terms of P content for herbivorous animals when the animal is no longer able to acquire sufficient P from its food to construct the P-rich materials needed for its body. An implication of this chain of mechanisms is that growth rate, body P content, and body RNA content should all be tightly correlated when growth rate is limited by dietary P intake, but when growth rate varies due to the impacts of some other factor (such as nitrogen or protein intake), growth and P content should no longer be correlated, as is seen in algae when cell quota of nonlimiting nutrient becomes decoupled from equilibrium growth rate (Droop 1974). Indeed, DeMott et al. (1998) showed a close correlation between body growth rate and body P content in *Daphnia magna* when grown on foods with elevated C : P ratio. However, he did not measure RNA, and so the mechanisms proposed here cannot be directly evaluated using their data. Finally, the proposed association of total RNA content and growth rate presents an obvious practical solution to the assessment of *Daphnia* growth (and thus fitness) in various field and laboratory conditions, since RNA measured as a percent of dry mass or RNA : DNA ratio has been shown to be correlated with growth rate in various organisms (algae, Dortch et al. 1983; bacteria, Koch 1970; invertebrates, Saiz et al. 1998; and fish larvae, Buckley 1984). Observations for *Daphnia* include those of Vrede et al. (2002), who argued that, if properly calibrated to species, stage, and temperature, RNA : DNA ratio can provide a snapshot index of zooplankton growth rate under natural conditions, making in situ incubations unnecessary. The prospects of such applications have now improved given the development of highly sensitive and reliable methods of RNA quantification in small samples (Gorokhova and Kyle 2002; Vrede et al. 2002).

Thus, the GRH predicts a tripartite set of close correlations among growth rate, RNA content, and P content under conditions in which growth rate is limited by the overall level of ribosome allocation and where RNA is an important sink for P. However, these associations should be broken under conditions under which P is not limiting for growth. While various observations consistent with the GRH have appeared (Main et al. 1997; DeMott et al. 1998; Gorokhova et al. 2002; Vrede et al. 2002), no detailed analyses of the entire set of correlations among growth, RNA content, and P content under diverse physiological conditions have yet been performed. In particular, it is still uncertain whether close coupling among growth–RNA–P will hold when N/protein is the limiting factor. We sought to illuminate these issues via a series of experiments with two species of *Daphnia* (*D. pulicaria*, *D. galeata*), measuring growth rate, RNA content, body nutrient content, and C : N : P ratios at various food conditions.

Methods

Cultures and chemostats—Laboratory experiments were conducted using *Daphnia galeata* and *Daphnia pulicaria*, collected at Lake Biwa, Japan, and established in stock cultures at Arizona State University (Tempe, Arizona).

The green alga *Scenedesmus acutus* was cultured in chemostats under four conditions of various P and N supply. There were three food treatments in the P-limited experiments and four for N-limited experiments in which algal C : P and C : N ratios differed accordingly. Algae in the P-limited experiment involved P-rich *Scenedesmus* (high P) cultured in an artificial medium (COMBO) developed by Kilham et al. (1998), modified to contain 1000 μmol L^{-1} N and 50 μmol L^{-1} P (molar N : P ratio 20) at a dilution rate of 1.0 d^{-1}. *Scenedesmus* with moderate P content (medium P) were cultured in COMBO with 500 μmol L^{-1} N and 12.5 μmol L^{-1} P (N : P 40) at a dilution rate of 0.25 d^{-1}. Low-P *Scenedesmus* (low P) was cultured in COMBO with 5 μmol L^{-1} P and 500 μmol L^{-1} N (N : P 100) at a dilution rate of 0.12 d^{-1}. Chemostats were allowed to equilibrate a minimum of 2 weeks before the start of the experiments. A fourth type of chemostat (low N), used for N-limited experiments, was prepared with 200 μmol L^{-1} N and 40 μmol L^{-1} P (molar N : P ratio 5) and run with slower dilution rates and increased light intensity to induce N limitation of the *Scenedesmus*. From these four cultures food suspensions having different C : P and C : N were prepared, sometimes by mixing different culture outputs to bring food C : P and C : N to a desired level (Table 1). The concentrations and C : N : P ratios of algae from the chemostats and in the mixtures were monitored every other day throughout the experiment. Algal samples on filters were dried in an oven at 60°C and held in a desiccator until analysis for C and N content (using Perkin–Elmer model 2400 elemental analyzer) and P content (using persulfate oxidation followed by the acid molybdate technique; APHA 1998). *Scenedesmus* for P-limited experiments had biomass C : P ratios of approximately 110.2 $\pm$ 7.3 (high P; mean $\pm$1 SE of values of algae measured every day or every 2 d depending on the length of the experiment), 456 $\pm$ 20.7 (medium P), and 933.7 $\pm$ 23.6 (low P). *Scenedesmus* for N-limited experiments had molar C : N of 6.31 $\pm$ 0.35 (same as high P) or 18.22 $\pm$ 0.56 (low N). In addition, two food suspensions with intermediate C : N values of 9.0 $\pm$ 0.42 and 15.0 $\pm$ 0.49 were prepared by mixing the high and low C : N *Scenedesmus* in appropriate proportions (Acharya et al. in press).

Growth experiments—Laboratory experiments were conducted on a large number of neonates of *Daphnia galeata* and *Daphnia pulicaria* of approximately the same age, size, and condition. To collect a large cohort of individuals, a few animals from stock cultures were isolated and grown individually on high concentration (>1.5 mg C L^{-1}) of high-P food for several days until they began reproduction. About 80–100 third clutch neonates were separated and grown individually in 250-ml jars with sufficient, high-quality food

Table 1. Average C : N, C : P, and N : P ratios of food (algae; *Scenedesmus acutus*) at high-, medium-, and low-P conditions for P-limited and high-N, medium-1, medium-2 and low-N conditions for N-limited experiments.

		C : N		C : P		N : P	
	Foot type	Mean	SE	Mean	SE	Mean	SE
				(molar ratio)			
P limited	high P	5.64	0.46	103.51	4.15	18.74	1.68
	medium P	10.15	0.19	472.15	12.22	46.53	1.13
	low P	11.01	0.25	994.97	19.40	90.49	2.70
N limited	high N	5.61	0.24	87.00	2.22	15.49	1.21
	medium N 1	9.00	0.35	130.48	1.81	14.50	0.86
	medium N 2	15.00	0.71	217.47	2.52	17.00	1.34
	low N	18.22	0.85	264.25	5.22	18.00	1.37

until they began reproduction. These animals were then transferred to clean jars at regular intervals and neonates (within 24 h of birth) from the third and subsequent clutches of these animals were used for the experiments.

Individual growth—P-limited experiments examined effects of the three diets (high P, medium P, and low P) at two food levels on the growth rate and chemical composition of individual *Daphnia.* Because of practical limitations of the culturing apparatus, separate sets of experiments were performed using *Daphnia galeata* and *Daphnia pulicaria*; for each species, treatments for low and high food were also performed separately. Growth rate was estimated by measuring the rate of change of animal body mass using video image analysis. The advantage of estimating growth by image analysis is that the growth rate could be measured on an individual basis while keeping the animals alive for subsequent growth. Thus, growth rate could be assessed for the same animal. The body area of animals was measured by taking lateral images of each animal and then calculating the animal's projected body area using Image-Pro Express®, Version 4 (Media Cybernetics) software. After image capture, animals were then transferred to 70-ml jars (one individual per jar) containing the appropriate food suspension and placed on a plankton wheel; a random subset of animals was put aside for later determination of initial dry mass and body C, N, P, and RNA contents (*see below*). Approximately 90% of the animals were in good condition after image capture; injured animals were discarded and replaced immediately. For each species in the P-limited experiments, there were a total of six treatment combinations: three diet type treatments (high-P, medium-P, and low-P *Scenedesmus*) supplied at low (0.25 mg C L^{-1}) and high (1.5 mg C L^{-1}) food levels. There were 12 replicates for each treatment combination. The animals used for weight regressions (*see below*) were also used for final P (four replicates of 15–20 animals each on GF/F filters), CHN (three replicates of 10–12 animals each in tin capsules), and RNA–DNA (seven replicates of one animal each in Eppendorf tubes) analysis. The N-limited experiments examined effects of the four types of food (*Scenedesmus* with low, two intermediate, and high C : N ratios) at high food concentration only. Growth rates of the animals in this experiment were determined as in the P-limited case, and animals were also sampled for CHN, P, RNA, and DNA analyses. There were a total of 18 replicate animals in each treatment: four animals used for determination of RNA–DNA, four for determination of P, and the remaining 10 animals were pooled into two replicates of five animals each for CHN analysis. The samples for CHN were also used for dry weight measurements in calculating C and N content.

Food and media were replenished every 24 h by hand transferring the *Daphnia* into fresh media. All growth experiments were conducted at a constant temperature (25°C) and a light : dark cycle of 14 : 10 h. Final measurement of body size was made after 72 h using the same image capture approach as the initial measurements. To avoid the confounding effect of shifts in allocation between growth and ovary development after the age of 5–6 d (DeMott 2003), we measured growth rates up to day 4 so that all the food was spent on growth only, not on reproduction. After the final image capture, animals were transferred to sample containers and placed in liquid nitrogen (RNA samples) or dried (C, N, P samples) for later analysis. Initial and final body area measurements were converted to body dry mass (*see below*) for calculation of growth rate (μ: in units of d^{-1}) as

$$\mu = \ln(\text{final weight}/\text{initial weight})/3$$

Weight–area regression—Simultaneous with the experiments just described, a second experiment was performed to establish the relationship between body area (from image analysis) and body mass for each treatment for the P-limited experiment. This was necessary because food quantity and food quality can affect the mass per unit length (and presumably area) in *Daphnia* (Hessen 1989; DeMott et al. 1998). From the large cohort of *Daphnia* neonates from which the other animals were collected, 8–10 animals each were placed in replicate 500-ml jars containing food of the appropriate quality and quantity combination. A few animals were randomly removed for initial measurements. These animals were photographed and placed immediately on preweighed filters for dry weight measurement. Animals remaining in the 500-ml jars were transferred to new food and media every 24 h by filtering each bottle through a cylinder with Nitex mesh at the bottom. During incubation, jars were kept gently agitated to prevent settling of food. After 72 h all the animals were photographed and placed onto preweighed filters. The dry weight of the samples and the body area measurements were used to create a regression between

Table 2. Results of analysis of variance (ANOVA) for average growth rates (d^{-1}), carbon content (%), phosphorus content (%), nitrogen content (%), RNA and DNA contents (%), of *D. pulicaria* and *D. galeata* at high and low food concentrations (P-limited experiments) and *D. pulicaria* at high food concentrations (N-limited experiment). The symbols **, *, m, and n.s. indicate highly significant ($p<0.01$), significant ($0.01<p<0.05$), marginally significant ($0.05<p<0.10$), and nonsignificant ($p>0.10$) effects, respectively.

Analysis of variance	*p* values							
	μ	C	P	N	C:P	N:P	RNA	DNA
P-limited experiments								
Species	n.s.	**	n.s.	**	m	*	**	**
Food concentration	**	n.s.	**	**	n.s.	n.s.	**	**
Food quality	**	m	**	n.s.	**	n.s.	**	*
Species × food quality	**	**	n.s.	n.s.	n.s.	n.s.	n.s.	**
Species × food concentration	*	n.s.	n.s.	n.s.	n.s.	m	n.s.	n.s.
Food quality × food concentration	m	n.s.	n.s.	n.s.	m	n.s.	n.s.	m
Species × food quality × food concentration	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s
N-limited experiments								
Food quality	**	n.s.	*	n.s.	m	*	**	**

animal body area and dry weight for each treatment for use in calculating growth rate and body nutrient and nucleic acid contents for animals raised in the main part of the experiment. All the weight–area regressions were linear and all had $p < 0.0001$ and $R^2 > 0.88$. In the N-limited experiment, the relative differences among the slopes of different relationships (high, medium 1, medium 2, and low N) were modest, but in the P-limited case slopes differed significantly between high and low food concentrations. In general, animals fed high-P food tended to have more weight per area than others. Specific parameters for these weight–area relationships are not presented here because they are specific to the microscopic apparatus employed in our laboratory and not useful for application elsewhere.

C, N, P, and RNA analysis—The P contained in individual animals was determined using colorimetric analysis after persulfate digestion (APHA 1998) and converted to P content (percentage of dry mass) using the body area–dry mass regression appropriate for the experimental treatment. Animal C and N content were determined similarly on dried and reweighed samples using a Perkin–Elmer elemental analyzer. The RNA and DNA contained in individual animals were determined using the microfluorometric method of Gorokhova and Kyle (2002) modified to more accurately measure DNA by use of DNase and converted to content measures (percentage of dry mass) using the appropriate body area–dry mass regression. The content of P contained in RNA was calculated from the RNA content data by assuming that nucleic acids are 9% P by mass (Sterner and Elser 2002).

Results

Food C:P and C:N ratios (Table 1) for P-limited and N-limited *Scenedesmus* chemostats remained fairly consistent throughout the experiments. Bulk C:P ratios in the medium-P and low-P treatments were above 300, the threshold elemental ratio for P limitation of *Daphnia* (Urabe and Watanabe 1992). Animals in the low-P food treatment suffered modest (~7%) mortality during the course of the experiment, but in general survivorship was good across all P-limited treatments. Animals in the N-limited experiment had 100% survivorship.

P-limited experiment—A combined analysis of variance indicated that growth rate differed for the two species, for different food types (high P, medium P, low P), and for different food concentrations (high vs. low) and that there were various significant interaction effects (Table 2). In general, both species grew better when food concentration was high (Fig. 1A). Similarly, both species did well on P-rich high-P food but grew more slowly on P-deficient medium- and low-P food. In addition, *Daphnia galeata* grew at the same rate as *D. pulicaria* at high concentrations of good quality food but had a higher growth rate on the medium-P and low-P foods. However, *D. pulicaria* had generally higher growth rates than *D. galeata* at low concentrations of food for all high-, medium-, and low-P food conditions. Thus, there were significant two-way interaction effects on growth rate for species–food concentration and species–food quality and a marginally significant effect for the interaction of food quality and quantity (Table 2).

Daphnia percentage C and N as dry mass for both species were significantly affected by the treatments (Fig. 1B; Table 2), but the effects were relatively modest. Animals fed low-P food generally had higher percentage C than animals fed high-P food (Fig. 1B), but these differences were marginally insignificant ($p \approx 0.06$; Table 2). Food quality had no discernible effect on percentage N. Food concentration had a significant effect on percentage N but not on percentage C (Table 2). There were no interaction effects among species, food quality, and food quantity for percentage N, but there was a marginal two-way interaction between food quality and food concentration for body C content. P content did not differ for the two species ($p > 0.47$ for effect of species; Table 2) but there was a significant overall effect of both food quality and quantity on body P content. Body P contents were higher in animals grown on high-P than on medium- and low-P algae (Fig. 1C; Table 2), and animals fed abundant food generally had higher P content than animals

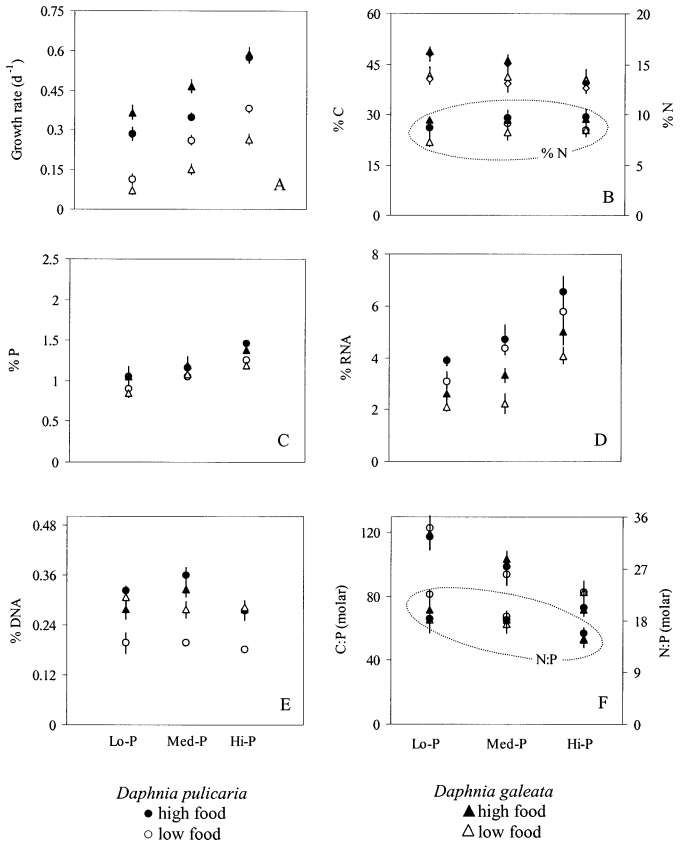

Fig. 1. (A) Average growth rates (d^{-1}), (B) carbon and nitrogen contents (percentage), (C) phosphorus contents (percentage), (D) RNA content (percentage), (E) DNA content (percentage), and (F) C : P and N : P (molar) ratios of *Daphnia pulicaria* and *Daphnia galeata* at high and low food concentrations in the P-limited experiment. Error bars are standard errors.

fed low quantity of food. There were no significant two-way interaction effects. However, *Daphnia* RNA content as a percent of dry mass also differed for the two species (Fig. 1D; Table 2), since *D. pulicaria* generally had higher percentage RNA at both high and low food concentrations than *D. galeata.* Food treatments significantly affected RNA content (Table 2), since both species generally had higher percentage RNA when fed good quality food at high concentration (Fig. 1D). DNA content differed for the two *Daphnia* species (Fig. 1E; Table 2); *D. galeata* had higher percentage DNA than *D. pulicaria.* Food quantity also affected DNA content, while the effect of food quality was marginally significant (Table 2), but there was a highly significant species–food quality interaction (Table 2). Finally, there was a marginally significant ($p = 0.07$) food quality–food concentration interaction effect on DNA content.

Chemical data were also generated for the neonates used in the experiments (data not shown). Relative to late juveniles (>72 but <96 h old) raised on high concentrations of high-P food, neonates of both species generally had lower percentage C (37.8% vs. 40.03%) and higher percentage N (10.25% vs. 9.08%) and percentage P (1.75% vs. 1.32%). They also had considerably higher RNA (7.71% vs. 5.34%) and DNA (0.72% vs. 0.25%) contents. Within each of the four combinations of species and food concentration, the data show close associations of growth with P and RNA contents and of P content with RNA–P content (Fig. 3 and Table 3; these data include values for neonates). When all the data from the P-limited experiment were analyzed together, they also demonstrated very strong overall relationships of RNA and P contents with growth (RNA vs. μ, $p < 0.0001$, $R^2 > 0.52$; P vs. μ, $p < 0.01$, $R^2 > 0.82$) and of total P content with RNA–P content ($p < 0.0001$, $R^2 > 0.85$). It is important to note that the close correlations between RNA–P content and overall P content are not an outcome of autocorrelation due to shared variable of body dry weight. As for other such analyses (Elser et al. 2003), similarly strong correlations are observed when paired data for RNA per individual and P per individual, matched for ontogenetic and physiological condition, are examined ($p < 0.003$, $R^2 > 0.85$ for the *Daphnia* data reported here).

Effects on body C : P and N : P ratios (Fig. 1F) generally were an inverse pattern relative to effects on body P content. Food quality and quantity had no effect on *Daphnia* body N : P ratios (quality, $p > 0.38$, and quantity, $p > 0.1$; Table 2) but did have an effect on body C : P (quality, $p < 0.001$, and quantity, $p < 0.01$; Table 2). Both species of *Daphnia* grown on the low-P diet had an average C : P (molar) ratio of about 120, but on high-P food the C : P ratio decreased to ~75. When we plotted between *Daphnia*'s body C : nutrient versus food C : nutrient (log–log: Fig. 5A), we found a positive linear relationship (slope ~0.18, $p < 0.01$, $R^2 = 0.93$).

N-limited experiment—Relative to responses to food C : P in the P-limited experiment, *D. pulicaria* in the N-limited experiment showed relatively modest (but statistically significant) differences in growth rates for diets with high and low C : N ratios (Fig. 2A; Table 2, $p < 0.0001$). Growth rate of *Daphnia* was highest when molar C : N ratio was six and decreased as dietary C : N ratio increased, especially from 9

Table 3. Regression coefficients for relationships between percentage P versus μ (d^{-1}), percentage RNA versus μ (d^{-1}), and percentage P versus percentage RNA-P of *D. pulicaria* and *D. galeata* at high and low food concentrations (P-limited experiments). All relationships were statistically significant ($p<0.02$).

Food level	Species	%P vs. μ (d^{-1})			%RNA vs. μ			%P vs. %RNA-P		
		Slope	*y* intercept	R^2	Slope	*y*-intercept	R^2	Slope	*y* intercept	R^2
High	*D. pulicaria*	1.49	0.50	0.99	10.81	1.46	0.97	1.62	0.62	0.98
	D. galeata	1.38	0.66	0.99	8.80	1.50	0.99	1.48	0.47	0.99
Low	*D. pulicaria*	1.72	0.76	0.91	10.78	1.06	0.87	1.66	0.58	0.92
	D. galeata	1.33	0.73	0.98	10.04	1.88	0.99	1.60	0.36	0.99

to 15. However, it is important to note that these responses are modest when compared to the reduction of growth rate in response to increased C : P ratio in the P-limited experiment. There were no significant differences in C or N content (Fig. 2B) of animals from the different N-limited foods ($p > 0.95$ for C and $p > 0.46$ for N; Table 2). Thus, the relationship between log(body C : N) versus log(food C : N) was not significant (Fig. 5B; slope ~0.03, $p > 0.05$). However, percentage P (Fig. 2C) and percentage RNA (Fig. 2D) as dry mass increased when C : N increased from 6 to 9 and peaked at 15 before decreasing at 18 ($p < 0.03$ for P and $p < 0.001$ for RNA, Table 2). Thus, unlike in the P-limited experiment, *Daphnia* in N-limited treatments showed an elevated percentage P and percentage RNA concomitant with a *decrease* in growth rate in these treatments. Indeed, no significant relationships between growth rate and RNA and P contents were observed (Fig. 4A,B; $R^2 = 0.13$ and 0.05, and $p > 0.57$ and 0.05, respectively), but it is important to note that only a modest range of growth rates was induced in the N-limited experiment compared to that achieved in the P-limited experiment. However, P and RNA–P still retained a significant relationship (Fig. 4C; $R^2 = 0.59$ and $p < 0.05$).

Discussion

Our data from the P-limited experiments are strongly supportive of the growth rate hypothesis: both RNA and P contents were positively and linearly related to growth rate; overall P content was strongly correlated with RNA–P content; and RNA itself also contributed a substantial fraction of overall biomass P. As noted by Elser et al. (2003), our results are consistent with similar cases of growth-dependent variation in RNA content found in *Escherichia coli* (Makino et al. 2003), cyanobacteria (Lepp and Schmidt 1998), yeast (Aiking and Tempest 1976), algae (Rhee 1978), and other species of *Daphnia* (DeMott et al. 1998; Vrede et al. 1998; Gorokhova et al. 2002) and with cross-species comparisons of P content and growth rates of crustacean zooplankton (Main et al. 1997). The linear relationships observed between *Daphnia*'s growth rate and P content in our experiments (Fig. 3A; Table 3) are quantitatively similar to those presented by DeMott et al. (1998) and Vrede et al. (1998). While the two *Daphnia* species in our experiments differed in absolute level of body RNA content, both species had similar positive linear relationships of RNA and P content with growth rate (Fig. 3), and the combined relationships for both species and food treatments put together were also very close. Furthermore, for both species, the P contained in RNA comprised a significant proportion of total body P (49% on average across all observations), and there was a tight relationship between percentage body P and percentage RNA–P (Fig. 3C; Table 3). Thus, our data for these two species of *Daphnia* under conditions of P-limited growth reveal tight three-way correlations among total P content, RNA content, and growth rate. These associations represent excellent examples of a syndrome that seems to be broadly applicable across diverse biota including crustaceans, insects, and microbes and involving both cross-species comparisons as well

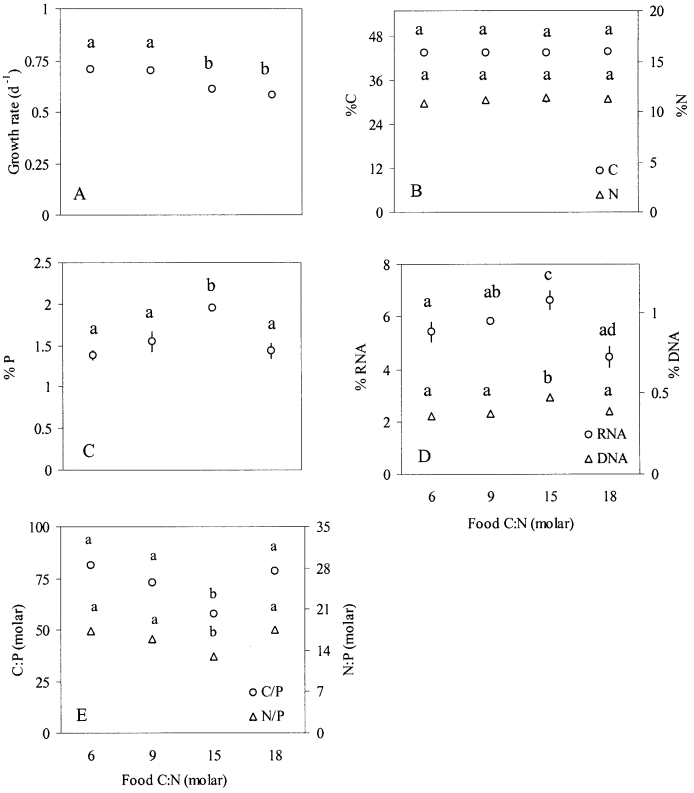

Fig. 2. (A) Average growth rates (d^{-1}), (B) carbon and nitrogen contents (percentage), (C) phosphorus content (percentage), (D) RNA and DNA contents (percentage), (E) C : P and N : P (molar) ratios of *Daphnia pulicaria* in the N-limited experiment. Lower case letters (a, b, c, d) in the figures represent results of Scheffe's comparison test. Error bars are standard errors.

as variations due to physiological or ontogenetic status (Elser et al. 2003).

There were, however, some discrepancies in these associations, since a relatively higher percentage of RNA was not associated with higher growth rate in every case. For example, *D. pulicaria* grown at low food concentration had a higher RNA content than *D. galeata* grown on high food, but *D. galeata* had a higher growth rate than *D. pulicaria* in this pair of treatments (Fig. 1A,D). This suggests that there may be an interspecific variation in the proportion of ribosomal RNA in the pool of total cellular RNA, since ribosomal RNA itself is thought to be tightly coupled to growth rate (Elser et al. 2000).

The N-limited experiment suggests somewhat of a different picture with respect to associations among growth rate, RNA content, and P content. Similar to the response to high C : P food in the P-limited experiment, *Daphnia* fed algae with high C : N ratio grew more slowly (but this response was modest, *see below*). However, while growth rate slowed as food C : N increased from 6 to 15, body P and RNA content increased until finally decreasing when food C : N was highest (18), even though growth rate did not change appreciably. Thus, growth rate seemed to be decoupled from RNA and P contents in this set of treatments. Indeed, there was no significant relationship between P content and growth rate

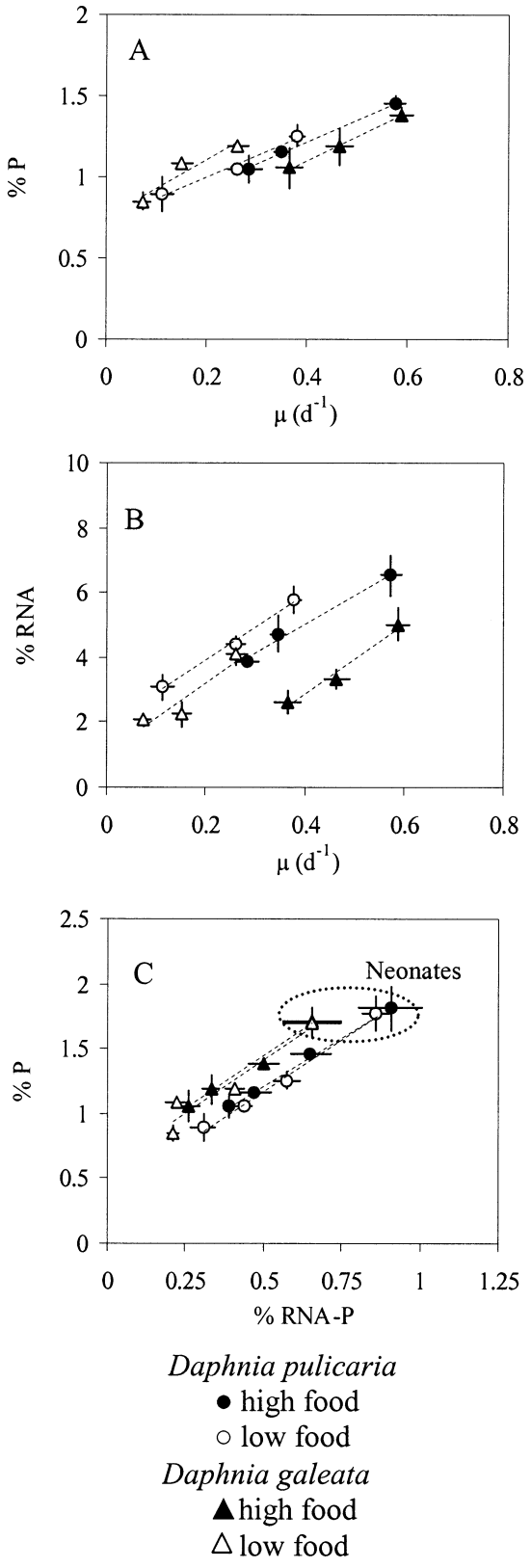

Fig. 3. Relationships between (A) percentage P versus μ (d^{-1}), (B) percentage RNA versus μ (d^{-1}), and (C) percentage P versus percentage RNA–P of *Daphnia pulicaria* and *Daphnia galeata* at high and low food concentrations in the P-limited experiment. Regression coefficients are given in Table 3. Error bars are standard errors.

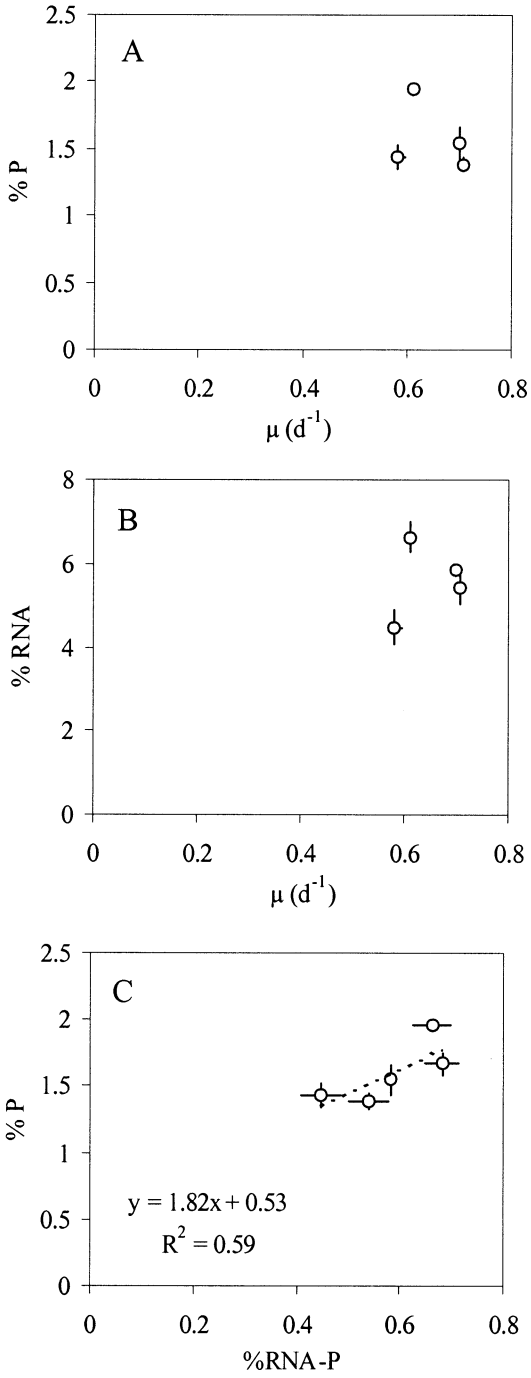

Fig. 4. Relationships between (A) percentage P versus μ (d^{-1}), (B) percentage RNA versus μ (d^{-1}), and (C) percentage P versus percentage RNA–P of *D. pulicaria* in the N-limited experiment. Error bars are standard errors.

(Fig. 4A; $p > 0.57$, $R^2 = 0.13$) nor between RNA content and growth rate (Fig. 4B; $p > 0.70$, $R^2 = 0.05$). However, there was still a significant correlation between total body P content and P content due to RNA (Fig. 4C, $p < 0.05$, $R^2 = 0.59$), although this relationship's slope and intercept were higher than observed for this relationship in the P-limited experiment. However, the lack of relationship between RNA

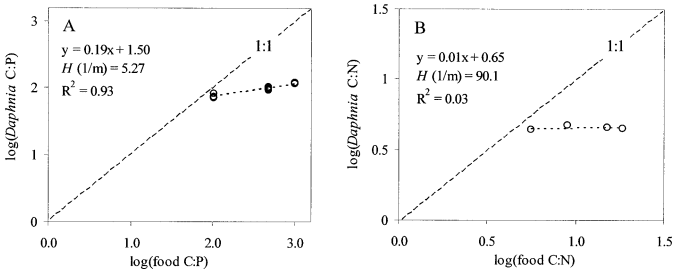

Fig. 5. Relationships between log(food C : nutrient) and log(*Daphnia* body C : nutrient) for (A) C : P ratio in *D. galeata* and *D. pulicaria*, high and low food concentrations pooled together in the P-limited experiment (analysis of variance [ANOVA], $p < 0.01$) and (B) C : N ratio in *D. pulicaria* in the N-limited experiment (ANOVA, $p > 0.05$). H (slope^{-1}) is the homeostatic regulatory coefficient. Error bars are standard errors.

and P contents with growth rate in the N-limited experiment should be interpreted cautiously, since our N-limited growth conditions did not induce a very wide range of growth rates in the animals, especially when compared to the P-limited case. Our data on *Daphnia* in the N-limited experiment have been placed in a broader context in the analyses of Elser et al. (2003), who noted a decoupling of growth, RNA, and P contents in other biota under certain conditions: in bacteria when substrate C : P ratios are low (Makino et al. 2003), under very low food conditions in *Daphnia* (Sterner unpubl. data), as well as in cyanobacteria and eukaryotic algae (Rhee 1978; Healey 1985). Thus, we hypothesize that under conditions of growth variation when P is in excess, the close coupling of growth with RNA and P contents is broken and a greater percentage of total body P should be contained in pools other than RNA. This is supported by the weak correlations in our N-limited case (but note the caveat above) and the fact that non-RNA pools comprised ~68% of the total body P in the N-limited experiment versus ~50% in the P-limited experiment. However, a more effective test for this decoupling would require that an equivalent range of growth rates be imposed, perhaps achieved by offering the animals more severely N-limited algae with even higher C : N ratios. For example, algal C : P ratios in the P-limited experiment ranged ~10-fold, but C : N ratios in the N-limited experiment varied only by a factor of three.

To the extent that the weak coupling observed in the N-limited experiment is real, the contrasting patterns of association between growth, P, and RNA from the P-limited and N-limited experiment have implications for application of RNA measurements to assess growth rates in nature. First, taken with the data summarized by Elser et al. (2003), it appears that relatively high RNA content does not necessarily indicate high growth rate if growth rates are constrained by high food C : N ratio or very low food abundance, but there is a very tight association if food C : P ratio is relatively high and P is likely limiting. Thus, our results do suggest that assessment of the RNA content of individual *Daphnia* should be a reliable way to assess growth rate in lakes where food abundance and C : P and N : P ratios are relatively high (that is, P is likely to be limiting). Second, as mentioned in the introduction, a variety of previous studies have proposed using the RNA : DNA ratio to assess field growth rates for various Metazoa as well as other groups. This option is attractive since it only requires nucleic acid assessment and not dry weight correction, itself challenging given the high lability of RNA molecules. However, it is important to note that we observed very close associations of growth rate with RNA content (percentage of dry mass contributed by RNA) and *not* with RNA : DNA ratio (data not shown, but inspect DNA and RNA data in Fig. 1). Thus, for *Daphnia* at least, reliable estimations of growth rate under appropriate conditions likely require steps to determine dry masses of the individuals involved. Third, our joint data on P and RNA contents may also help in further interpreting data from field-collected animals. That is, the fact that *Daphnia* growing more slowly due to high food C : P ratio carried a greater fraction of their total body P in RNA than when growth was limited by high food C : N or very low food abundance (Elser et al. 2003) suggests that the discrepancy between total P content and P contributed by RNA for field-collected animals, properly paired for body size, might be used as an index of stoichiometric limitation due to elevated food C : P ratio. That is, when a high percentage of body P is contributed by RNA, one would infer that there is limitation by P, but when a larger unexplained fraction is observed, some other factor, such as N or overall food abundance, may be limiting to growth.

Our data also bear on the issue of stoichiometric homeostasis. To date, stoichiometric theory has operated with the assumption that consumers maintain fixed body elemental composition despite variation in the elemental composition of their food (Andersen 1997; Loladze et al. 2000; Sterner and Elser 2002). However, previous studies have shown that *Daphnia*'s P content declines when raised on food with low P content (Sterner et al. 1993; DeMott et al. 1998). We obtained similar results, since body P content and C : P ratio both changed significantly when animals were raised on foods with different C : P ratios. However, it is important to point out that this body variation was relatively modest in comparison with the range of variation presented in the food: body P content and C : P ratios for animals raised on low C : P versus high C : P food differed less than twofold even though food C : P ratio varied tenfold. Following Sterner and Elser (2002), we calculated the homeostatic regulatory coefficient H for C : nutrient ratio based on the log–log regression of body C : nutrient ratio and food C : nutrient for each species in the two experiments. In this analysis, H is the inverse of the slope of the relationship and thus varies from 1 (no homeostasis, if body elemental composition exactly tracks variation in the food) and infinity, if there is strict homeostasis and there is no variation in body stoichiometry in response to variation in the diet. For C : P, estimates of H for *D. galeata* at high and low food concentrations were 4.8 and 6.1 (respectively), and for *D. pulicaria* they were 4.4 and 6.4, relatively high and indicative of strong, but not strict, homeostasis in body C : P ratio. We performed a similar analysis for body C : N ratio in the N-limited experiment. The H value was very high (~90 for *D. pulicaria* at high food concentration), since indeed there was no significant effect of food C : N ratio on animal C : N. Thus, an assumption of strict homeostasis appears invalid for C : P but not

C : N ratio in response to variation in the nutrient content of the diet. However, body C : P ratio responded linearly to variation in food C : P ratio (Fig. 1F), which suggests that modifications of growth equations in stoichiometrically explicit food web models (e.g., Andersen 1997; Loladze et al. 2000) may relax the strict homeostasis assumption in a relatively simple manner by introducing a linear dependence of body P content proportional to growth rate when food P content falls below the threshold elemental ratio.

We also observed that P content in the food still played an important role in *Daphnia* growth even at lowered food abundance. Both species of *Daphnia* in our experiments had lower growth rates at low concentrations of P-limited food than at low concentrations of P-rich food. Similarly, Boersma and Kruetzer (2002) demonstrated effects of dietary P content at low food concentrations (0.03–0.15 mg C L^{-1}) for *D. magna*, in contrast to Sterner and Robinson's (1994) earlier report for *D. obtusa*, where they observed no effect of P content at low food quantity (0.025–0.3 mg C L^{-1}). In our P-limited experiments, juvenile *Daphnia* fed high-P high concentration food (1.5 mg C L^{-1}) had the highest growth rate, followed by medium- and low-P high concentration foods. A similar linear growth gradient with food C : P was observed at low food concentration (0.25 mg C L^{-1}). Furthermore, correlations among growth and P content (described below) were as strong under subsaturating food as under high food (Fig. 3) for both species, indicating that P limitation remained important even in the low food treatment. Thus, while our experiments did not involve food levels close to the starvation threshold, our results do support the idea that effects of stoichiometric food quality are not confined to conditions of saturating food abundance. However, from our data we cannot evaluate whether the effects of food P content diminish when food concentrations approach the starvation threshold, as imposed in Sterner and Robinson's original study and in the case reported in Elser et al. (2003), where *Daphnia pulex*'s RNA and P contents were uncorrelated with growth rate when food levels were extremely low (0.1 mg C L^{-1}).

Our results build on the foundation laid by Elser et al. (1996, 2000) by showing that, in these two species of *Daphnia* at least, there is indeed a very tight association among growth and P content and that this association is directly the result of variation in relative allocation to P-rich RNA. Elser et al. (2003) show that these associations extend to a wide array of biota. Our work also helps delineate the conditions under which these mechanisms operate: close associations are to be expected when growth rate is proportional to the relative abundance of the ribosome machinery, and ribosomal RNA synthesis itself is limited by the acquisition of P from the diet (in the case of animals) or environment (in the case of bacteria and algae). However, when growth rate is constrained by other factors, such as the high dietary C : N ratio in our N-limited experiment, the close associations are broken since the rate-limiting step in growth likely shifts from ribosome production itself to other steps in the growth process. In the case of N-limited growth, the constraint likely shifts to a limitation of protein synthesis (translation) itself due to shortages of amino acids or amino acid precursors. Delineating the nature and generality of these metabolic responses as animals shift from P- to N-limited or energy-limited growth will be important in deciding how (and whether) to incorporate such biological constraints into stoichiometrically explicit ecological and evolutionary models that are now emerging.

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