

## Associations among ribosomal (r)DNA intergenic spacer length, growth rate, and C:N:P stoichiometry in the genus *Daphnia*

Lawrence J. Weider<sup>1</sup> and Karen L. Glenn

The University of Oklahoma Biological Station, HC-71, Box 205, Kingston, Oklahoma 73439

Marcia Kyle and James J. Elser

Department of Biology, Arizona State University, Tempe, Arizona 85287-1501

### Abstract

The growth rate hypothesis (GRH) proposes that variation in organismal C:N:P ratios is associated with growth rate due to differential allocation to P-rich ribosomal RNA to support growth. A further extension of the GRH proposes that this variation is associated with the structure of ribosomal (r)DNA genes (e.g., promoter–enhancer sequences in the rDNA intergenic spacer [IGS]) that influence the production rate of rRNA. To test these ideas, we examined growth rate, rDNA structure, RNA:DNA ratios, and elemental composition (C:N:P) in multiple clones of three *Daphnia* species (*pulex*, *pulicaria*, *magna*) in controlled laboratory experiments. Results show that a portion of the rDNA motif, consisting primarily of the IGS, shows considerable interspecific and intraspecific length variation. Significant interspecific differences in all parameters were found, while significant intraspecific (clone) effects were detected only for RNA:DNA ratios and growth rate. As predicted by the GRH, there were significant positive correlations between RNA:DNA ratio and either growth rate or rDNA/IGS length, and a significant positive correlation between rDNA/IGS length and growth rate, when clonal means for all three species were examined. However, no clear-cut relationships between RNA:DNA and either C:P or N:P were observed for any of the three species. Likewise, no discernible intraspecific relationships between growth rate and either C:P or N:P were detected. These preliminary findings suggest that further study of elemental composition, nucleic acid content, and growth rates of organisms may lead to a clearer understanding of the role of stoichiometric constraints in influencing the ecology and evolution of natural populations.

All organisms need to deal with the allocation of resources to the competing needs of somatic growth, maintenance, and reproduction. How organisms reach a balance between these competing needs has resulted in the evolution of a wide variety of life-history strategies (Stearns 1992). The underlying genetic/biochemical and/or physiological mechanisms that operate here are often studied separately from any consideration of the role that the nutritional/feeding environment has on influencing growth and/or reproduction (Elser et al. 1996). However, recently a new approach to such questions has emerged in the form of ecological stoichiometry (Elser et al. 1996, 2000; Sterner and Elser 2002), which focuses on the balance of energy and multiple chemical elements in ecological interactions (Sterner and Elser 2002). In this approach, studies examine differences in elemental carbon (C):nitrogen (N):phosphorus (P) ratios among different trophic

levels and taxa, and how these differences influence and reflect the structure and dynamics of biological levels of organization spanning genes to ecosystems (Elser et al. 2000; Sterner and Elser 2002). Sterner and Elser (2002) summarize the basic concepts and principles related to this burgeoning field, which incorporates some of the basic approaches developed in resource ratio competition theory (Tilman 1982) for autotrophs competing for inorganic nutrients, but emphasizing relationships between trophic levels (e.g., herbivores and their food items, Elser and Hassett 1994). These interspecific and trophic-level interactions are then examined in relation to ecosystem processes such as energy transfer in food webs and nutrient recycling dynamics (Sterner et al. 1992; Urabe et al. 2002).

Much stoichiometric work has focused on herbivorous zooplankton (Elser and Hassett 1994), particularly in the genus *Daphnia* (Urabe et al. 1997; Vrede et al. 2002) because of the keystone role that *Daphnia* plays in aquatic food webs, as a major grazer on algae and bacteria and recycler of the potentially limiting nutrients nitrogen (N) and phosphorus (P). Interestingly, *Daphnia* appears to be among the most P rich of common zooplankton found in lakes (Hessen and Lyche 1991; Elser et al. 2000). To date, most of this work has centered on examining interspecific differences (Main et al. 1997), while virtually no systematic study of intraspecific (interclonal) differences has previously been conducted (Elser et al. 2000).

Recent work has been progressing on elucidating the linkage between the biomass C:N:P stoichiometry of biota and

<sup>1</sup> Corresponding author (ljweider@ou.edu).

### Acknowledgments

We thank Lynnette Matthews, Robyn Moran, and Amy Ramsey for laboratory assistance. This work was supported by a grant from NSF's Integrated Research Challenges in Environmental Biology (IRCEB) program (NSF-9977047). We benefited from numerous discussions with our IRCEB colleagues, especially Theresa Markow, Teresa J. Crease, and Tom Watts. We thank Nelson G. Hairston Jr. and two anonymous reviewers for constructive criticisms of an earlier version of the paper. Finally, we thank Teresa J. Crease for designing the PCR primers and providing us with the sequences used in the rDNA IGS analyses.

cellular/subcellular phenomena such as allocation to ribosomal (r)RNA and how variation in rDNA structure might be associated with the evolution of key life-history traits such as organismal growth rates (Elser et al. 2000). Specifically, this "growth rate hypothesis" (GRH) (Elser et al. 1996, 2000) proposes the following: (1) faster growing organisms should have higher RNA content and RNA:DNA ratios than slower growing organisms and (2) as a result, rapidly growing organisms should have higher levels of P (i.e., low C:P and N:P ratios) in their bodies than slower growing organisms because of the increased allocation to P-rich RNA. Elser et al. (2000) have extended the hypothesis to the genetic level, arguing that fast growth rate and high RNA allocation demand high rates of RNA production, and thus particular variations in the genes coding for ribosomal RNA (the rDNA) are expected. In particular, rDNA intergenic spacer (IGS) length should be positively correlated with growth rate because the longer the IGS, the greater the number of potential promoter, enhancer, and terminator sites in the different subrepeats and, thus, a greater potential for transcription (i.e., rRNA production) supporting ribosome production needed for rapid growth.

Few direct tests of this hypothesis have been made (see Elser et al. 2000 for a synthetic review of evidence related to individual components of this hypothesis), although a recent study by Gorokhova et al. (2002) involving artificial selection within a single clone of *Daphnia pulex* as a model system has found direct support for a number of the predictions of the GRH. In the present paper, we extend the testing of the GRH to multiple clones within multiple species of *Daphnia* under nonselective conditions.

## Materials and methods

We collected and isolated animals and established laboratory clonal cultures of three *Daphnia* species: *D. pulex* (four clones; Px-1, Px-2, Px-3, and Px-4), *D. pulicaria* (three clones; Pul-1, Pul-2, and Pul-3), and *D. magna* (three clones; M1, M2, and M3). Clones were chosen because they represented a fairly broad range in intergenic spacer (IGS) length variation (see below). *D. pulex* were isolated from a shallow (<0.5 m), ephemeral pond (LL4) in northwestern Iowa (43°23'N, 95°11'W), while *D. pulicaria* and *D. magna* (clones M1 and M2) were isolated from Brant Lake, South Dakota (43°58'N, 96°56'W), and Round Lake, South Dakota (44°58'N, 96°49'N), respectively. *D. magna* clone M3 was isolated from the Grösser Binnensee (54°20'N, 10°38'E), a slightly brackish water, shallow (<2 m) coastal lake in northern Germany, adjacent to the Baltic Sea. Brant Lake and Round Lake had salinities/conductivities of 790 and 260, seston C:P ratios (atomic) of 60.2 and 99.5, seston N:P ratios of 9.7 and 12.6, and maximum depths of 3 m and 4 m, respectively. No data are available on the physicochemical features of pond LL4, and Lampert (1991) describes the Grösser Binnensee more fully.

Clonal isolates were raised in the laboratory in a standard artificial medium called COMBO (Kilham et al. 1998) and fed the chemostat-raised green alga, *Scenedesmus obliquus*, at a concentration of 3 mg carbon L<sup>-1</sup>. Three to four gravid

females per 100-ml jar with multiple (i.e., 4–12) jars per clone were checked daily for release of offspring. Offspring of known age (i.e., within 24 h) were pooled separately for each clone and then separated into replicate (i.e., 3–8) 600-ml jars (i.e., approximately 100–150 neonates per jar) containing COMBO and fed 3 mg carbon L<sup>-1</sup> of *S. obliquus* that is grown at low (atomic) N:P ratio (~17:1) at high growth rate and thus has low (atomic) C:P (~140) and (atomic) N:P (~20) ratios (Kilham et al. 1998). Previous studies (e.g., Sterner 1993) have indicated that this alga grown under these culture conditions can support rapid *Daphnia* growth. Medium and food were changed daily. Cohorts from each clone were harvested on day two and day four for separate CHN and P analyses (see below). Numbers of individuals were counted, placed into preweighed aluminum boats, dried for a minimum of 48 h (60°C), and held in a desiccator until dry mass could be obtained using a Cahn 27 automatic ultra-electrobalance.

Additional individual 2-day-old and 4-day-old animals were measured (i.e., body lengths) to the nearest 0.04 mm using a Zeiss dissecting microscope, placed in 10 µl of RNAlater (Ambion Inc.), and frozen at -20°C until RNA and DNA analyses were conducted (see below).

Juvenile growth rates were determined as  $\mu$  (d<sup>-1</sup>) = ln (final body mass of 4-day-old cohort/initial mass of 2-day-old cohort)/2. (Cohort masses were corrected for number of animals per cohort.) For each clone, the number of replicates ranged from 4 to 12 cohorts, depending on the ability to synchronize the cohorts.

*Elemental and biochemical analyses*—The analysis of phosphorus (P) followed a modified persulfate digestion method and the ascorbic acid method of Franson (1998). The analysis of carbon (C) and nitrogen (N) followed standard protocols using a Europa Scientific (Northwich, U.K.) model ANCA-SL isotope ratio mass spectrometer. Elemental contents were expressed on a percent dry mass basis from which atomic ratios for C:P, C:N, and N:P were calculated. (Replicates ranged from 3–8 per clone per analysis.) We followed the basic extraction and quantification methods of Gorokhova and Kyle (2002) and Kyle et al. (2003) for analysis of RNA and DNA contents of individual or small numbers of *Daphnia*.

*IGS length variation*—Two sets of oligonucleotide primers were used in conjunction with the polymerase chain reaction (PCR) to amplify the entire intergenic spacer (IGS) region of the rDNA array using either a Perkin-Elmer 9700 thermal cycler or a MJ Research PTC-200 cycler. PCR reactions (50 µl total volume) consisted of the following components: 0.3–0.5 µmol L<sup>-1</sup> per primer per reaction, 10 mmol L<sup>-1</sup> Tris-HCl (pH 8.3), 50 mmol L<sup>-1</sup> KCl, 1.5–2.0 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 2 mmol L<sup>-1</sup> of each dNTP, approx. 2.5 units of *Taq* polymerase, and 10–1000 ng of template DNA. For *Daphnia pulex* and *D. pulicaria*, IGS38 forward (5'-GTT-TAG-ACC-GTC-GTG-AGA-CAG-GTT-AG-3') and 3102R reverse (5'-ATA-CCA-AAG-TTT-TTG-CGT-CG-3') primers were used to amplify an approximately 3–4 kilobase (kb) fragment of the IGS, which flanks the highly repetitive region. Owing to differentiation (i.e., sequence divergence) in the rDNA array

for *D. magna*, a second set of primers was designed and used: IGS29 forward (5'-GTG-AGC-TGG-GTT-TAG-ACC-GTC-GTG-AG-3') and 18s418R (5'-CGT-TTC-TCA-GGC-TCC-CTC-TCC-GG-3'). For the IGS38/3102R primer pair, the following PCR thermal cycling parameters were used: one cycle of initial denaturation at 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, annealing at 60°C for 30 s, extension at 70°C for 4 min, followed by 10 cycles of 94°C for 30 s, annealing at 60°C for 30 s, extension at 70°C for 4 min 20 s (20 s per cycle autoextension), and a final 70°C extension for 7 min. For the IGS29/18s418R primer pair, the following PCR thermal cycling parameters were used: an initial denaturation cycle at 94°C for 1 min, followed by 20 cycles of 94°C for 20 s and annealing/extension at 68°C for 6 min, followed by 10 cycles of 94°C for 20 s and annealing/extension at 68°C for 6 min 20 s (20 s per cycle autoextension).

PCR products were subjected to agarose gel electrophoresis using 1.0% AMERSCO (Solon, Ohio) agarose, run at 3–4 cm h<sup>-1</sup>, stained with ethidium bromide (4 µg ml<sup>-1</sup>), and visualized using a Kodak electrophoresis documentation and analysis system (EDAS) 290 digital camera and Kodak 1D image analysis system software. Molecular weight markers were run alongside PCR products to permit sizing of IGS length variants.

**Statistical analyses**—All statistical analyses were conducted using Statistix version 7.0 (Analytical Software <http://www.statistix.com>). Simple linear regression analyses were conducted, as well as nested analysis of variance (ANOVA). Percentage P, C:P (atomic), %N, N:P (atomic), %RNA, and RNA:DNA were measured for 2-day-old and 4-day-old cohorts for each clone within a given species. The means of the two age cohorts for each parameter, along with growth rates, were analyzed using nested ANOVAs with clones nested within species. (Note: we took mean values for each parameter for the combined age cohorts because these mean values more fully reflect the average physiological state of the animals during the course of the study, thus reducing any bias as a result of potential age effects.) Owing to the unbalanced design of having unequal numbers of clones per species (i.e., four clones for *Daphnia pulex*, three clones each for *D. magna* and *D. pulicaria*), it was necessary to calculate nested ANOVAs using all three clone combinations for the four *D. pulex* clones. We report the mean values for these analyses (Table 1). To control for type I experiment-wide error, a sequential Bonferroni procedure for multiple simultaneous tests was performed (Statistix).

## Results

**Nested ANOVAs**—Results from the nested ANOVAs of interspecific differences and clonal differences (nested within species) in %P and %N revealed significant interspecific differences for both parameters, while significant clonal differences were only detected for %N (Table 1). Bonferroni multiple comparison tests revealed that for %P, *pulicaria* = *pulex* > *magna*, while for %N, the opposite pattern was observed (i.e., *magna* > *pulex* > *pulicaria*).

An examination of stoichiometric ratios (C:P and N:P)

Table 1. Results of nested ANOVAs for analysis of mean combined values (i.e., 2- and 4-day-old *Daphnia*) for %P, %N, C:P (atomic), N:P (atomic), %RNA, RNA:DNA, and growth rate.

Parameter	Level	$F_{2,6}$	df	$p$
%P	A	48.80	18, 26	0.00001**
	B	2.12		0.1022
%N	A	166.39	32, 40	0.00001**
	B	3.42		0.0102*
C:P (atomic)	A	262.30	18, 26	0.00001**
	B	3.93		0.011*
N:P (atomic)	A	128.23	18, 26	0.00001**
	B	1.01		0.4504
%RNA	A	24.75	30, 38	0.00001**
	B	1.67		0.2927
RNA:DNA	A	38.32	30, 38	0.00001**
	B	5.14		0.0011**
Growth rate	A	6.22	60, 68	0.0040**
	B	11.62		0.00001**

\* Significant at  $p < 0.05$ .

\*\* Significant using the Bonferroni correction ( $0.05/7 = p < 0.008$ ).

Levels: species (A); clones nested within species (B). Degrees of freedom (df) = residual, total.

revealed significant interspecific differences in C:P and N:P (Table 1), while significant clonal differences were only found for C:P (Table 1). Bonferroni multiple comparison tests revealed that for both ratios *magna* > *pulex* > *pulicaria*. Thus, in general *D. pulex* and *D. pulicaria* were more P rich than *D. magna*.

Analyses of RNA:DNA ratios (RNA:DNA hereafter) and %RNA revealed significant interspecific differences for both parameters, with significant clonal differences only found for RNA:DNA (Table 1). Bonferroni multiple comparisons revealed that for %RNA, *pulex* > *magna* = *pulicaria*, while for RNA:DNA, *pulex* > *magna* > *pulicaria*.

Nested ANOVA results for growth rates indicated both significant interspecific and clone (nested within species) differences (Table 1), with *magna* = *pulex* > *pulicaria*.

**Associations and regression analyses**—No clear-cut patterns of association between RNA:DNA ratios and either C:P (atomic) or N:P (atomic) were observed (Fig. 1), when comparing the clonal means for these parameters across the three species (Fig. 1a, RNA:DNA vs. C:P, df = 8,  $r^2 = 0.078$ , *n.s.*; Fig. 1b, RNA:DNA vs. N:P, df = 8,  $r^2 = 0.090$ , *n.s.*). Likewise, comparisons of growth rates versus either C:P (atomic) or N:P (atomic) ratios (Fig. 2) shown no discernible relationship within species. Further, no significant correlations were found, when data for all clones across all species were combined (Fig. 2a, growth vs. C:P, df = 8,  $r^2 = 0.133$ , not statistically significant; Fig. 2b, growth vs. N:P, df = 8,  $r^2 = 0.105$ , not statistically significant).

*A posteriori* testing indicated that *D. pulicaria* clone Pul-2 exhibited a very low growth rate in relation to its rDNA IGS length. Results from Grubbs' test for outlier detection (Grubbs 1969) indicated that the critical Z value of 2.29 ( $n = 10$ ) was exceeded by the test Z value (2.35) at  $p < 0.05$ , indicating that clone Pul-2 represented a significant outlier and therefore should be omitted from further analyses.

With the omission of *D. pulicaria* clone Pul-2, we found

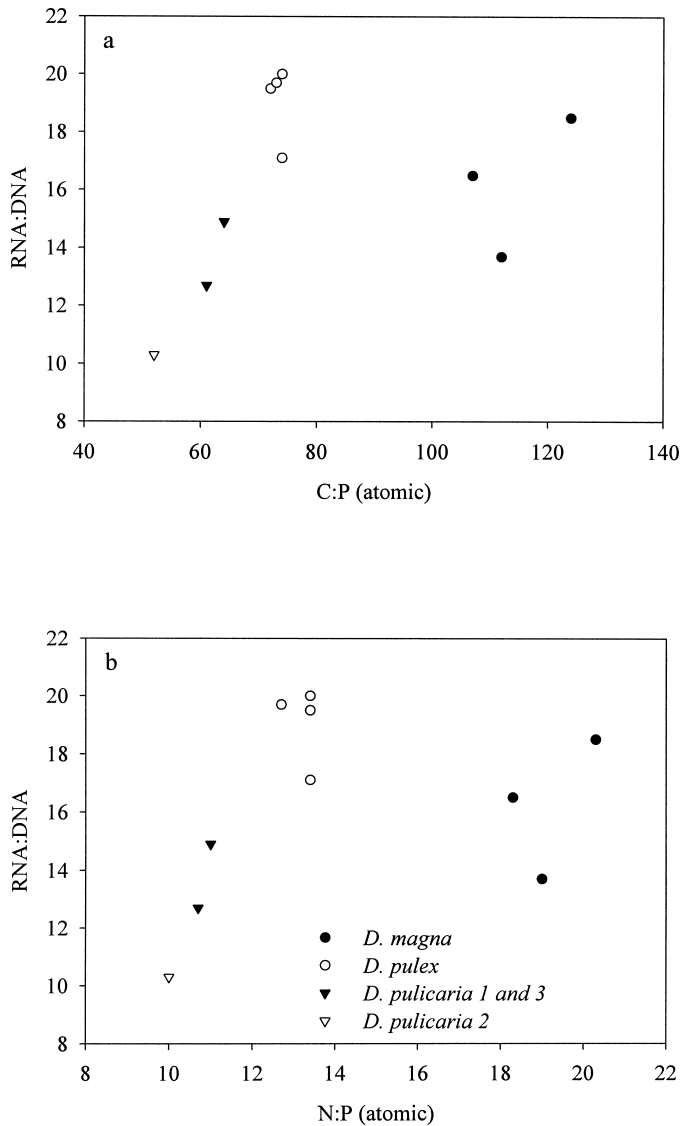


Fig. 1. (a) Plot of RNA:DNA versus carbon:phosphorus (C:P atomic) ratio; (b) plot of RNA:DNA versus nitrogen:phosphorus (N:P atomic) ratio. Clonal means for each of the 10 clones in the study are given: *D. magna* (M1, M2, M3); *D. pulex* (Px-1, Px-2, Px-3, Px-4); *D. pulicaria* (Pul-1, Pul-2, Pul-3).

a number of relationships that were consistent with the predictions of the GRH. There was a significant positive correlation between RNA:DNA ratios and growth rate for the combined data set (Fig. 3;  $df = 7$ ,  $r^2 = 0.527$ ,  $p < 0.05$ ). Further, a significant correlation was found between rDNA IGS length and RNA:DNA, when clonal means for the three species were analyzed together (Fig. 4;  $df = 7$ ,  $r^2 = 0.628$ ,  $p < 0.05$ ). Finally, a significant positive relationship between rDNA IGS length and growth rate for these three *Daphnia* species was observed ( $df = 7$ ,  $r^2 = 0.63$ ,  $p < 0.02$ , Fig. 5).

## Discussion

Results presented here suggest that a number of associations among RNA:DNA, rDNA structure (i.e., IGS length),

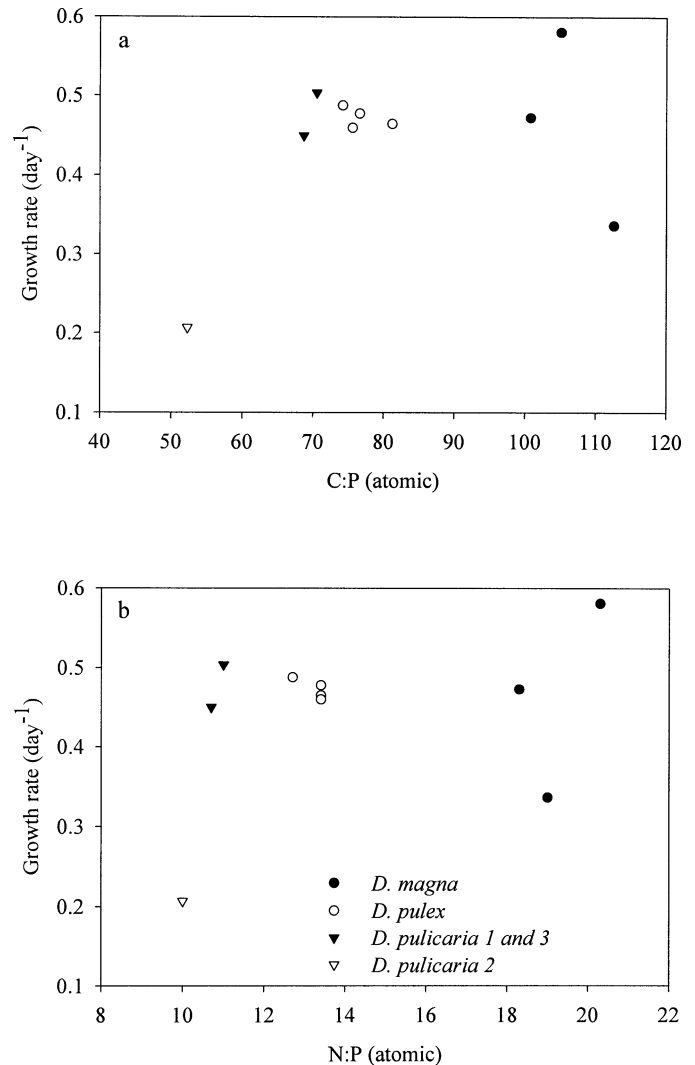


Fig. 2. (a) Plot of growth rate,  $\mu$  (d<sup>-1</sup>) versus carbon:phosphorus (C:P atomic) ratio; (b) plot of growth rate,  $\mu$  (d<sup>-1</sup>) versus nitrogen:phosphorus (N:P atomic) ratio. See Fig. 1 legend for details.

body stoichiometry (C:P or N:P), and growth rates in *Daphnia* exist. For example, when the data from the clonal means of all three species are combined to compare RNA:DNA ratio and specific growth rate, a significant correlation was found (Fig. 3), thus lending support to a prediction of the GRH (i.e., that there should be a positive association between RNA:DNA and growth rate). Further, results from the rDNA IGS, RNA:DNA ratio, and growth rate comparisons indicated a significant positive correlation between rDNA IGS length and RNA:DNA ratios (Fig. 4), as well as a significant positive correlation between rDNA IGS length and growth rate (Fig. 5). However, discrepancies were also noted. For example, we did not observe any clear-cut relationships between RNA:DNA and either C:P or N:P ratios for any of the three *Daphnia* species (Fig. 1). In fact, one might argue that if any patterns were to be discerned from these relationships it would be a positive association between RNA:DNA and either of these stoichiometric ratios

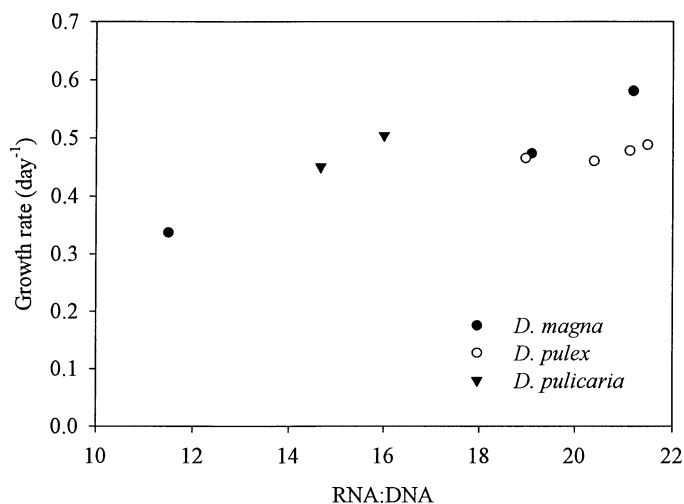


Fig. 3. Plot of growth rate,  $\mu$  ( $\text{d}^{-1}$ ) versus RNA:DNA ratio. Note: *Daphnia pulicaria* is represented by only clones Pul-1 and Pul-3. See Fig. 1 legend and text for details.

for *D. magna* and *D. pulicaria* (Fig. 1), the exact opposite conclusion that would be predicted by the GRH. The third species, *D. pulex*, showed no discernible pattern at all (Fig. 1).

For the comparisons between growth rates and either C:P or N:P (Fig. 2), no discernible relationships within species could be detected, most likely because of the small numbers of clones examined.

In general these results are inconclusive regarding the existence of associations among growth rate, RNA, and P contents in these daphnids. Given the observation of quite close associations among these parameters in published studies of interspecific variations in zooplankton (Main et al. 1997) and ontogenetic and physiological variations in crustaceans, insects, and bacteria (Elser et al. 2003), we suspect that our study presents a relatively weak test of the GRH as the clones and species examined presented only a relatively narrow range of growth rates across which to examine variation in C:N:P and RNA:DNA ratios (0.3–0.5  $\text{d}^{-1}$  vs. a range of 0.1–1.2  $\text{d}^{-1}$  in the study of Main et al. 1997).

Our genetic data were more supportive of the relevant components of the GRH (Elser et al. 2000), as noted above for the rDNA IGS, RNA:DNA ratio, and growth rate comparisons (Figs. 3, 4, and 5). Clones having longer IGS length variants generally exhibited higher growth rates. The mechanisms involved in these associations are not entirely clear. However, previous work using *Drosophila* (Cluster et al. 1987) and *Daphnia pulex* (Gorokhova et al. 2002) has suggested that a likely mechanism is that longer IGS variants have more subrepeats (each containing a promoter, enhancer, and terminator site for transcription) and thus can maintain a higher production rate of ribosomal RNA during rapid growth. Further testing of this IGS length–growth rate relationship is warranted.

**Role of rDNA IGS length heterogeneity**—The intergenic spacer (IGS) region of rDNA is the most variable and polymorphic region within the rDNA motif (Crease 1993). With-

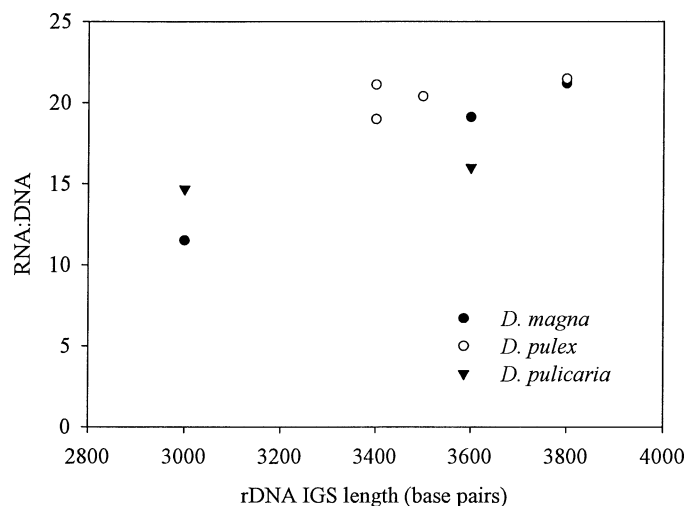


Fig. 4. Plot of rDNA IGS length (bp) versus RNA:DNA ratio. See Fig. 1 and 3 legends for details.

in the genus *Daphnia* (particularly among species of the subgenus *Daphnia*, i.e., *D. pulex*, *D. pulicaria*), the IGS contains two highly repetitive regions consisting of 330-basepair (bp) subrepeats and 200-bp subrepeats, separated by approximately 50 nucleotides (nt) of unique sequence (Crease 1993). It is believed that promoter, enhancer, and terminator sites for transcription are located within each of these 330-bp subrepeats (Crease 1993), as has been shown in the subrepeat region of the IGS in other organisms (e.g. *Drosophila*, Cluster et al. 1987; maize, Rocheford et al. 1990). Crease (1993, 1995) has shown that length variation within the IGS region of *Daphnia pulex* is highly polymorphic, since multiple length variants are often present not only within single populations but also within single clones. Indeed, even individual daphnids can be heteroplasmic for IGS length variants.

A recent study by Gorokhova et al. (2002) highlights this variation and demonstrates its potential ecoevolutionary sig-

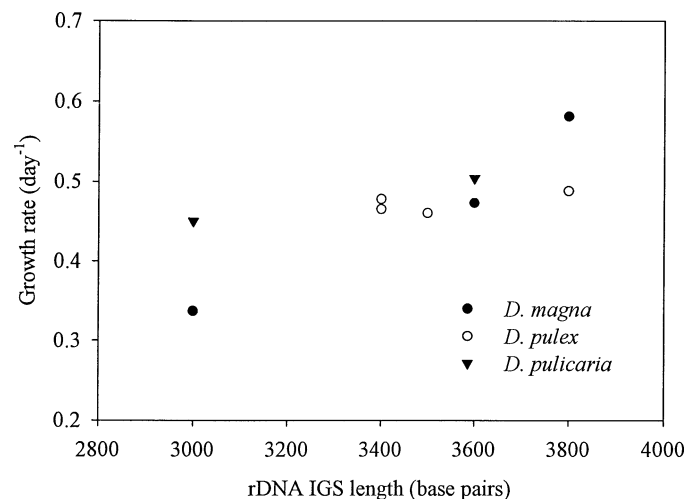


Fig. 5. Plot of growth rate,  $\mu$  ( $\text{d}^{-1}$ ) versus rDNA IGS length (bp). See Figs. 1 and 3 legends for details.

nificance. In their experiments, a single, obligately parthenogenetic clone of *Daphnia pulex* was subjected to artificial selection on production rate (i.e., fecundity). Significant divergence occurred within four generations and resulted in associated divergence of juvenile growth rate as a (negatively) correlated character. This was, in turn, associated with differential distribution and expression of different IGS length variants that appear to be directly related to differences in these key life-history features. These authors postulated that long spacers may provide an advantage, which is manifested via greater rDNA transcriptional efficiency and higher rates of ribosome production, leading to increased rates of protein synthesis, thus enhancing growth and/or developmental rates. Further, these results indicate that even among asexual (parthenogenetic) organisms like *Daphnia*, there is considerable potential for rapid adaptive change affecting major life-history characters such as reproductive output and growth rate. Significant associations among IGS variants, juvenile growth rate, juvenile RNA content, and juvenile P content were all observed in this study.

The present study reinforces these findings, as we document interspecific and intraspecific (interclonal) variation in stoichiometric ratios, RNA:DNA ratios, IGS length variation, and growth rates. What these preliminary results suggest is that a number of the basic tenets/predictions of the growth rate hypothesis (GRH) appear to be at least partially supported (although certainly more species and more clones should be tested to more fully evaluate the robustness of these relationships) and that there may be an intrinsic link between the stoichiometric demands of organisms and critical biochemical/molecular structures (i.e., nucleic acids such as RNA) that affect key organismal processes such as specific growth rate. We are also coming closer to an understanding of the genomic basis of these associations.

*Implications for population and community structure*—Similar to our findings, earlier work by Main et al. (1997) noted species-specific differences in the within-species relationships between growth rates and stoichiometric ratios for various zooplankton taxa (including daphnids). Specifically, these authors found that for *D. lumholtzi*, growth rate was positively correlated with %P and negatively correlated with N:P. However, these same relationships did not hold true for two other *Daphnia* species, *D. magna* and *D. obtusa*. Thus, it appears that different species have different approaches for allocating resources among growth and biochemical pools containing P. This may have the effect of avoiding stoichiometric bottlenecks at certain times of development (e.g., Villar-Argaiz and Sterner 2002), with the additional consequence of obscuring patterns of growth-RNA-P coupling at the scale of within-species comparisons. It is conceivable, then, that clones/genotypes within individual populations of *Daphnia* are influenced by the severity of stoichiometric food quality limitation in the field. This could be tested by examining the responses of clones differing in IGS length to field supplementation of dietary P (e.g., Elser et al. 2001) over a season during which seston C:P is changing.

We also document significant variation in C:P and N:P ratios among different species of *Daphnia* (Table 1). Ander-

sen and Hessen (1991) and Hessen and Lyche (1991) have shown previously that different species of *Daphnia* can have different N:P and %P levels in their body tissues, with some values of %P varying by as much as 40% between species (e.g., *D. galeata*, 1.11% P; *D. rosea*, 1.80% P).

Such findings suggest that species with differing P demands may be favored in environments that differ in stoichiometric food quality, as has been postulated and documented by Andersen and Hessen (1991), Sterner et al. (1992), and Elser et al. (1996), among others. Such differences suggest evolutionary constraints among members of this genus and require further study with additional species/isolates.

Likewise, we detected intraspecific (interclonal) differences in growth rates for two of the three species, *D. magna* and *D. pulicaria* (Table 1). Such intraspecific (genetic) variation fuels the microevolutionary process leading to potential shifts in the competitive dominance of different clones (genotypes) and ultimately to population genetic changes through time. As pointed out by Elser et al. (2000), these types of links between suborganismal processes (i.e., growth rate) and higher level (i.e., population, community, ecosystem) processes influence the evolutionary potential of genotypes/clones and species and, thus, establish a mechanistic basis of reciprocal feedbacks between ecosystem conditions and microevolutionary change.

*Future directions*—It is clear from the present preliminary study that much additional work is needed to clarify the potential associations among growth, nucleic acid metabolism, and C:N:P ratios that our limited data have suggested. More species and clones need to be examined, in particular, members of the third subgenus of *Daphnia*, the *Hyalodaphnia*, to determine the robustness of these findings. Owing to limitations with the available IGS primers (i.e., lack of intraspecific resolution), we were unable to screen members of this subgenus. More sophisticated work is also needed to unravel the specific mechanisms connecting rDNA structure, RNA production, and rates of protein synthesis, ultimately leading to impacts on organismal growth rates. The crude measure of rDNA IGS length variation is a case in point. It is still not clear whether IGS length per se is the important parameter to be examining (Reeder 1984). Most likely, beyond the mere multiplicity of promoter-enhancer repeats that influence IGS length, the underlying structure (sequence) of the rDNA motif is also critical in influencing the transcriptional process (Reeder 1984), as has been suggested by other authors in such systems as maize (Rocheford et al. 1990) and wheat (Sardana et al. 1993). Such information is generally lacking for *Daphnia*.

Future work should therefore focus on a higher resolution study of the underlying rDNA motif structure, which would involve sequencing of the IGS region and potentially the examination of patterns of methylation (i.e., effects on gene expression; Sardana et al. 1993) and transposable element load (e.g., gene silencing by retro-transposons; Sullender and Crease 2001). Such genomic-level studies coupled with more extensive studies of growth rate and biochemical and C:N:P parameters should be a fertile field for future research efforts. This work would yield a much deeper under-

standing of the tempo and mode of evolutionary change within the functional framework of ecological stoichiometry.

## References

- ANDERSEN, T., AND D. O. HESSEN. 1991. Carbon, nitrogen, and phosphorus content of freshwater zooplankton. *Limnol. Oceanogr.* **36**: 807–814.
- CLUSTER, P. D., D. MARINKOVIĆ, R. W. ALLARD, AND F. J. AYALA. 1987. Correlations between development rates, enzyme activities, ribosomal DNA spacer-length phenotypes, and adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **84**: 610–614.
- CREASE, T. J. 1993. Sequence of the intergenic spacer between the 28S and 18S rRNA-encoding genes of the crustacean, *Daphnia pulex*. *Gene* **134**: 245–249.
- . 1995. Ribosomal DNA evolution at the population level: Nucleotide variation in intergenic spacer arrays of *Daphnia pulex*. *Genetics* **141**: 1327–1337.
- ELSER, J. J., D. DOBBERFUHL, N. A. MACKEY, AND J. H. SCHAMPEL. 1996. Organism size, life history, and N:P stoichiometry: Towards a unified view of cellular and ecosystem processes. *BioScience* **46**: 674–684.
- , AND R. P. HASSETT. 1994. A stoichiometric analysis of the zooplankton-phytoplankton interaction in marine and freshwater ecosystems. *Nature* **370**: 211–213.
- , H. HAYAKAWA, AND J. URABE. 2001. Nutrient limitation reduces food quality for zooplankton: *Daphnia* response to seston phosphorus enrichment. *Ecology* **82**: 898–903.
- , AND OTHERS. 2000. Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* **3**: 540–550.
- , AND OTHERS. 2003. Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* **6**: 936–943.
- FRANSON, M. A. H. (ED.) 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, American Water Works Association, Water Environment Federation.
- GOROKHOVA, E., T. DOWLING, L. J. WEIDER, T. J. CREASE, AND J. J. ELSER. 2002. Functional and ecological significance of rDNA IGS variation in a clonal organism under divergent selection for production rate. *Proc. R. Soc. Lond. B* **269**: 2373–2379.
- , AND M. KYLE. 2002. Analysis of nucleic acids in *Daphnia*: Development of methods and ontogenetic variations in RNA-DNA content. *J. Plankton Res.* **24**: 511–522.
- GRUBBS, F. E. 1969. Procedures for detecting outlying observations in samples. *Technometrics* **11**: 1–21.
- HESSEN, D. O., AND A. LYCHE. 1991. Inter- and intraspecific variations in zooplankton element composition. *Arch. Hydrobiol.* **121**: 343–353.
- KILHAM, S. S., D. A. KREGER, S. G. LYNN, C. E. GOULDEN, AND L. HERRARA. 1998. COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* **377**: 147–159.
- KYLE, M., T. WATTS, J. SCHADE, AND J. J. ELSER. 2003. A microfluorometric method for quantifying RNA and DNA in terrestrial insects. *J. Insect Sci.* **3.1**: 1–7.
- LAMPERT, W. 1991. The dynamics of *Daphnia magna* in a shallow lake. *Verh. Int. Ver. Limnol.* **24**: 795–798.
- MAIN, T. M., D. R. DOBBERFUHL, AND J. J. ELSER. 1997. N:P stoichiometry and ontogeny of crustacean zooplankton: A test of the growth rate hypothesis. *Limnol. Oceanogr.* **42**: 1474–1478.
- REEDER, R. H. 1984. Enhancers and ribosomal gene spacers. *Cell* **38**: 349–351.
- ROCHFORD, T. R., J. C. OSTERMAN, AND C. O. GARDNER. 1990. Variation in the ribosomal DNA intergenic spacer of a maize population mass-selected for high grain yield. *Theor. Appl. Genet.* **79**: 793–800.
- SARDANA, R., M. ODELL, AND R. FLAVELL. 1993. Correlation between the size of the intergenic regulatory region, the status of cytosine methylation of ribosomal RNA genes and nucleolar expression in wheat. *Mol. Gen. Genet.* **236**: 155–162.
- STEARNS, S. C. 1992. The evolution of life histories. Oxford Univ. Press.
- STERNER, R. W. 1993. *Daphnia* growth on varying quality of *Scenedesmus*: Mineral limitation of zooplankton. *Ecology* **74**: 2351–2360.
- , AND J. J. ELSER. 2002. Ecological stoichiometry: The biology of elements from molecules to the biosphere. Princeton Univ. Press.
- , ———, AND D. O. HESSEN. 1992. Stoichiometric relationships among producers, consumers, and nutrient cycling in pelagic ecosystems. *Biogeochemistry* **17**: 49–67.
- SULLENDER, B. W., AND T. J. CREASE. 2001. The behavior of a *Daphnia pulex* transposable element in cyclically and obligately parthenogenetic populations. *J. Mol. Evol.* **53**: 63–69.
- TILMAN, D. 1982. Resource competition and community structure. Princeton Univ. Press.
- URABE, J., J. CLASEN, AND R. W. STERNER. 1997. Phosphorus-limitation of *Daphnia*: Is it real? *Limnol. Oceanogr.* **42**: 1436–1443.
- , J. J. ELSER, M. KYLE, T. YOSHIDA, T. SEKINO, AND Z. KAWABATA. 2002. Herbivorous animals can mitigate unfavourable ratios of energy and material supplies by enhancing nutrient recycling. *Ecol. Lett.* **5**: 177–185.
- VILLAR-ARGAIZ, M., AND R. W. STERNER. 2002. Life history bottlenecks in *Diatomus clavipes* induced by phosphorus-limited algae. *Limnol. Oceanogr.* **47**: 1229–1233.
- VREDE, T., J. PERSSON, AND G. ARONSEN. 2002. The influence of food quality (P:C ratio) on RNA:DNA ratio and somatic growth rate of *Daphnia*. *Limnol. Oceanogr.* **47**: 487–494.

Received: 13 February 2003

Accepted: 26 August 2003

Amended: 16 September 2003