PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Increased risk of phosphorus limitation at higher temperatures for *Daphnia magna*

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Abstract Invertebrate herbivores frequently face growth rate constraints due to their high demands for phosphorus (P) and nitrogen (N). Temperature is a key modulator of growth rate, yet the interaction between temperature and P limitation on somatic growth rate is scarcely known. To investigate this interaction, we conducted a study on the somatic growth rate (SGR) of the cladoceran *Daphnia magna*, known to be susceptible to P-limitation. We determined the SGR across a broad range of dietary P content of algae (carbon (C):P ratios (125–790), and at different temperatures (10–25°C). There was a strong impact of both temperature and C:P ratio on the SGR of *D. magna*, and also a significant interaction between both factors was revealed. The negative effect of dietary C:P on growth rate was reduced with decreased temperature. We found no evidence of P limitation at lowest temperature, suggesting that enzyme kinetics or other measures of food quality overrides the demands for P to RNA and protein synthesis at low temperatures. These findings also indicate an increased risk of P limitation and thus reduced growth efficiency at high temperatures.

Introduction

A large number of experiments and field studies have demonstrated that consumers, most notably herbivores, can face dietary phosphorus (P) limitation (Sterner and Elser 2002). In essence, this effect is commonly due to a high carbon (C):P ratio in autotrophs relative to consumers demands. This implies that there will be an excess intake of C that needs to be disposed off, and which translates into reduced growth efficiencies (Hessen and Anderson 2008). Moreover, P deficiency may constrain the synthesis of ribosomes, thereby reducing protein synthesis and consequently somatic growth (Elser et al. 2000).

Most experiments and observations related to nutrient limitation have been conducted in a temperature range between 15 and 25°C, and there is scarce and somewhat mixed evidence for the relevance of stoichiometric regulation at low temperatures. For autotrophs, the content of P and N relative to C generally increase with increasing latitude, which is interpreted as a temperature response (Reich and Oleksyn 2004; Kerkhoff et al. 2005; Lovelock et al. 2007). Although the data are scarcer, the same tendency seems to also hold true for metazoans (Moore et al. 1996; Woods et al. 2003). Cold-adapted ectotherms show higher cell-specific levels of P and rRNA than individuals of the same species living under higher temperatures (Woods et al. 2003). This has been interpreted as a

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compensatory response to a reduced efficiency of protein synthesis at low temperatures, i.e. more ribosomes are needed to maintain a given protein synthesis rate at low temperatures.

The interactive effects of food quality and temperature can be very important in understanding *Daphnia* distribution and production in lakes (Cole et al. 2002). Vertically migrating *Daphnia* can be expected to experience major fluctuations in both temperature and food quality over short time spans (Sterner and Schwalbach 2001). Especially, pond-dwelling species like *D. magna* may experience strong both seasonal and diurnal fluctuations in ambient temperature. For species subject to short-term fluctuations in temperature (and possibly also food quality), high temperatures facilitating high somatic growth rates (SGR) would pose the highest dietary demands for P (and N) in order to maximize SGR. Conversely, low temperatures would make the nutrient quota of food less relevant since SGR will be constrained by low temperature. Temperate herbivores commonly face strong variability in temperatures diurnally, annually and spatially. Zooplankton may face a fivefold temperature range from spring to summer and from surface to deep water layers, and their stoichiometric responses over this temperature range remain to be settled.

Our study is aimed towards the assessment of short-term responses in changing food quality and temperature. To test for the interaction of temperature and P limitation, we performed a factorial study where the SGR of the cladoceran *Daphnia magna* was assessed over a broad range of dietary C:P ratios (C:P = 125–790), and at different temperatures (10, 15, 20, and 25°C). This species commonly inhabits small water-bodies with dramatic diurnal and seasonal changes in temperature, and it has been shown previously that this species is sensitive to C:P ratios in its food (cf. Hessen et al. 2002).

There are two possible outcomes from these experiments. (1) The effect of dietary P limitation on SGR is greatest at low temperatures. This would be expected if the lower protein synthesis rates at low temperatures are compensated by a greater number of ribosomes, which entails more RNA, and, thus, an increased demand for P (Elser et al. 2000). (2) The effect of dietary P limitation is greatest at high temperatures were the SGR is highest.

Materials and methods

The green algae *Selenastrum capricornutum* Printz was grown at 20°C in chemostats at a dilution rate of 0.2 day^{-1} (20% of the volume continuously replaced over 24 h) on COMBO medium (Kilham et al. 1998) with the phosphate concentration reduced to 2 μM. The cultures reached

steady states before experiment initiation. Details about the chemostats setup can be found in Hessen et al. (2002). We added several amounts of dissolved inorganic P (K_2HPO_4) to batches of the chemostat-grown, P-depleted, algae in order to "spike" them with P and lower their C:P ratio while keeping other food quality parameters constant (e.g., N, sterols, fatty acids). The P-depleted algae were incubated in darkness with the dissolved inorganic P for 30 min before we dispensed the algae into the new experiment vessels and onto filters for later measurements. About 1 h passed from when the inorganic P was added until the *Daphnia* were feeding on the spiked algae. The spiking procedure is based on the observation that P-depleted phytoplankton assimilate such inorganic P within a few minutes, yielding a shift in C:P (Rothhaupt 1995; Plath and Boersma 2001). We cannot completely rule out that there still were some changes in the macromolecular makeup of the algae during the course of the experiments, yet there are good reasons to assume that they should be minor compared with the direct P effects. The grazing experiments were performed in the absence of light to minimize macromolecular anabolism, and the chlorophyte used is practically devoid of key unsaturated fatty acids (cf. Leu et al. 2006). The spiking method is used as a published protocol designed for separating the effects of P and fatty acids (e.g., Rothhaupt 1995; Plath and Boersma 2001). See Table 1 for an overview of the C:P ratios of the algal diets. The algae were diluted to 3 mg C L^{-1} using N- and P-free COMBO medium before being used in the experiments. Algae culture C concentration for daily food dilution was calculated from the optical density (OD) at 633 nm using a previously established calibration curve between the OD

Table 1 The food types used in the experiments and the C:P-ratio variation within each type

Experiment ID	Way of preparation	Average C:P	Standard deviation	n
A	Spiking	792	183	5
A	Spiking	542	80	5
A	Spiking	471	109	5
A	Spiking	447	113	5
A	Spiking	383	95	5
A	Spiking	294	79	5
A	Spiking	187	73	5
A	50 μM P media	141	42	5
B	Spiking	736	161	10
B	Spiking	487	85	10
B	Spiking	164	62	10
B	50 μM P media	125	6	10

There were 5 replications (n) for each dietary treatment in experiment A (1 sample per day on five separate days), and 10 replications in experiment B (as in A but with 2 pseudoreplications each day)

and measured C concentration. Algae for C and P analysis were collected on pre-combusted (530°C, 3 h) GF/F filters (Whatman, Kent, UK). C contents were analyzed on a Thermo Finnigan FlashEA 1112 elemental analyzer. Samples for particulate P were analyzed using a modified molybdate blue method (Menzel and Corwin 1965) after persulphate digestion.

The strain of *Daphnia magna* Straus used in all experiments had been kept in the laboratory for many generations at 20°C. In nature, *D. magna* are typically found in pond populations that during summer may experience strong temperature fluctuations. In shallow rock-pools typically inhabited by *D. magna*, temperatures at noon may approach 30°C on sunny days, and decrease to <15°C during night.

We conducted two factorial experiments, denoted as "A" and "B", with either eight (A) or four (B) levels of algae P content (C:P = 125–790, mol:mol), and four temperatures (10, 15, 20, and 25°C). One of the food treatments was *S. capricornutum* grown in standard COMBO medium (50 μM inorganic P), the others were P-depleted algae with inorganic P added, which are referred to as "spiked" in order to separate the two types of algal food used. The C:P of these treatments [C:P = 125 (A) and 141 (B)] were close to those algae spiked to the lowest C:P ratios [C:P = 164 (A) and 187 (B)] (see Table 1). There were two replicates in experiment A, and three replicates in experiment B. Each replicate consisted of eight juveniles (from the second brood and <24 h old) transferred to a bottle with 50 mL of the treatment food. The dietary C:P ratios used ranged from less than the incipient limiting threshold for *Daphnia* (molar C:P 225–375 at 20°C; Brett et al. 2000) to ratios well above this threshold. Animals were moved to fresh food suspensions every 24 h. The experiments were run in darkness in temperature-controlled rooms.

Experiment A was terminated on day 6 and B on day 7, both before any eggs were deposited in brood pouches. B was carried out 1 day longer than experiment A in order to add one additional day of growth and to check if ontological development stage might disturb the growth rate pattern caused by diet and temperature. Within each experiment, the different temperature treatments where terminated on the same day.

The dry weights of individual *D. magna* were estimated using a regression relationship to pixel area on a digital image, according to Færøvig et al. (2002). SGR were calculated as $[\ln(w_t) - \ln(w_0)]/t$, where w_0 is the dry weight at time zero and w_t at time t. SGR were analyzed by linear models (lm-function in R) using temperature (continuous variable), C:P ratio in the food (continuous variable), food type (factor variable: spiked or not) and experiment ID (factor variable: A or B) as explanatory variables. A

starting model with all four explanatory variables and their pairwise interactions was optimized by step-wise removal of the least important variable or variable interaction, as long as this removal improved the model performance. The models were evaluated using the Bayesian Information Criterion (BIC; Johnson and Omland 2004) and the model with the greatest support (lowest BIC) was selected as the best (Crawley 2007). In order to confirm the parameter selection based on BIC, we checked the significance of food type (spiked or not) and the parameters in the final model using likelihood ratio tests. We checked the residuals for indications of non-linearity, normality and heteroscedasticity during the model evaluation process, and the final model passed all the standard assumptions for linear regression. Statistics were carried out in R (R Development Core Team 2009).

Results

D. magna SGR varied from 0.1 to 0.4 day^{-1} and was affected by both experiment temperature and diet (Figs. 1, 2 and 3). The final model, which included temperature, food C:P ratio, experiment ID, and an interaction between temperature and food C:P, explained 86% of the variance in *D. magna* SGR and was highly significant ($p < 2.2 \times 10^{-16}$; see Table 2 for additional information). Observed versus predicted SGR (Fig. 1a) and a quantile–quantile plot (Fig. 1b) of the residuals confirm that the residuals were approximately normally distributed with constant variance. The slight non-linearity was mainly due to a single data point, and not considered worth pursuing. For each observation i, the final model is given by:

$$\begin{aligned} SGR_i = \alpha &+ \beta_1 \times \text{temperature}_i + \beta_2 \times \text{diet C:P ratio}_i \\ &+ \beta_3 \times \text{experiment ID}_i \\ &+ \beta_2 \times \text{diet C:P ratio}_i \times \text{temperature}_i + \varepsilon_i. \end{aligned}$$

The parameters α and β are the population intercept and slope, unexplained information is captured by the residuals ε that are assumed to be normally distributed with an average of zero and a variance σ^2. Whether the food had been spiked with P or grown on a high P medium did not significantly affect the SGR and was not included as a factor in the final model (likelihood ratio test: $F_1 = 0.36$, $p = 0.55$). Both the diet × temperature interaction and experiment ID were higly significant (likelihood ratio tests: $F_1 = 18.7 \ p = 3.4 \times 10^{-5}$ and $F_1 = 20.7 \ p = 1.4 \times 10^{-5}$, respectively) and kept in the model.

D. magna SGR were dependent on both temperature and the diet C:P, with the highest SGR observed at high temperatures and low C:P ratios (Fig. 2; note that the lines in the figures are the model fit to the data and not

Table 2 Summary statistics for the final model explaining *Daphnia* somatic growth rates

Coefficients	Estimate	Standard error	t value	p
Intercept	-0.015	0.022	-0.70	0.49
Temperature	0.018	1.2×10^{-3}	15	$<2 \times 10^{-16}$***
C:P ratio	5.6×10^{-5}	4.8×10^{-5}	1.2	0.25
Experiment ID	-0.030	6.5×10^{-3}	-4.6	1.4×10^{-5}***
Temperature × C:P ratio	-1.1×10^{-5}	2.6×10^{-6}	-4.3	3.4×10^{-5}***

It explained 86% of the total variance, F statistics was 164.7 on 4 and 107 DF, the whole model p value was $<2.2 \times 10^{-16}$
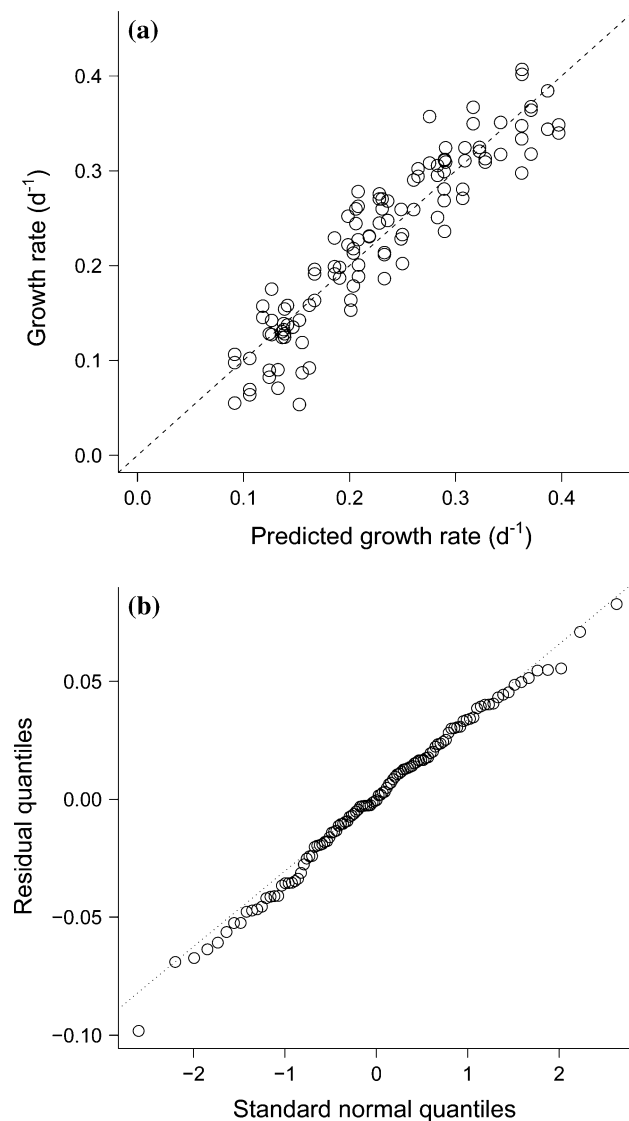
*** Variable significance at the <0.001 level

Fig. 1 **a** The somatic growth rate of *Daphnia magna* as predicted by the linear model plotted against the measured growth rates ($r^2 = 0.86$). The *dashed line* shows the 1:1 relationship. **b** Quantile–quantile plot

regression lines). The SGR was still positive at the lowest temperature, but owing to the small size increments the results were rather scattered at 10°C. There was also a significant interaction between temperature and diet C:P ratios, such that diet C:P affected *D. magna* SGR less at low temperatures (Table 2, Fig. 2). There was a significant difference between the two experiments, but this effect was purely additive with no interaction with the treatment variables and represented only 2.7% of the total variance in SGR. Thus, while SGR from experiment B were lower than in A, the general response patterns were identical. Figure 3 illustrates this in a contour plot of predicted SGR as function of temperature and diet C:P ratio (experiment ID effect set to A). Mortality losses were negligible in both experiments.

Discussion

There were effects of both temperature and nutritional C:P-ratio on *Daphnia* SGR, confirming general expectations, yet the interaction between the two is quite intriguing and clearly shows that temperature reaction norms are influenced by food stoichiometry, and that the effect of dietary P limitation varies with temperature. The slope of the relationship between SGR and dietary C:P became less negative with decreasing temperature, to the extent that there was practically no effect of food stoichiometry on SGR at low temperatures. This finding implies that in situ temperature needs to be taken into account when assessing stoichiometric food quality effect on natural zooplankton populations.

Although the results of the two experiments were very similar, the significant interaction between them suggests that other factors than temperature and diet may slightly modify the dynamics of the animals' growth. The instantaneous growth rate of developing *Daphnia* decrease with age, as reflected in RNA:DNA ratios (Gorokhova and Kyle 2002). The lower growth rates in experiment B are most likely an effect of this decreasing instantaneous growth rate as they grew for 1 day longer.

For temperate species like *D. magna*, our data suggest that while P deficiency can constrain SGR at most temperatures, enzyme kinetics or other aspects of food quality
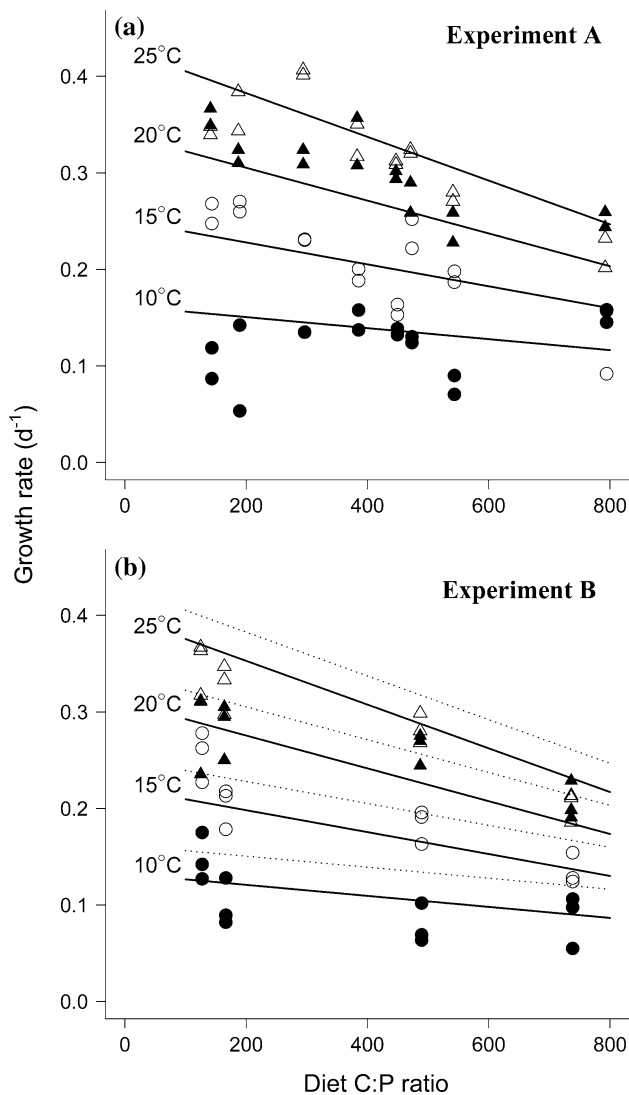
Fig. 2 *Daphnia magna* somatic growth rates on diets of different food algae C:P at four temperatures: 25°C (*open triangles*), 20°C (*solid triangles*), 15°C (*open circles*), 10°C (*solid circles*). **a** Observations from experiment A, the *solid lines* display the model fit at each of the four temperatures. **b** Observations from experiment B. The *solid lines* display the model fitted to the data from experiment B, and the *dotted lines* show the model fitted to experiment A. For model statistics, see Table 2

Fig. 3 Contour plot of predicted SGR as function of temperature and diet C:P ratio (based on experiment a). *Lines* represent combinations of diet C:P ratio and temperature that are predicted to yield the same SGR (isoline labels; day^{-1})

overrides the demands for P to RNA and protein synthesis at low temperatures. It has previously been demonstrated that growth and life history parameters in *Daphnia* generally did not respond to changes in temperature if food quantity was low, but did so when there were high food quantities (Orcutt and Porter 1984). This suggests that the temperature sensitivity of *Daphnia* may be similarly affected by reduced food quality as by reduced food quantity.

One possible concern is that food quality can affect the feeding rate of *Daphnia* (cf. Plath and Boersma 2001) and
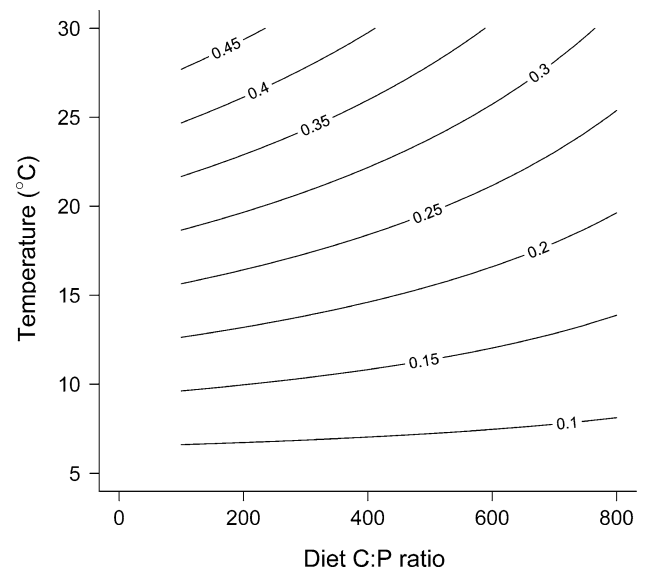
that the observed results could reflect that the available food supply was exhausted in the high C:P treatments, and thereby resulting in reduced growth due to food quantity limitation. However, previous studies using the same range of C:P, the same strain of *Selenastrum* and the same clone of *D. magna* have failed to demonstrate any significant effect on grazing rate owing to C:P (cf. Hessen et al. 2002; Darchambeau et al. 2003). It is, however, clear that grazing rate will be affected by temperature, and hence even if the grazing rate should be affected by C:P of the food, one could argue that this is part of the observed net response of grazer performance along the food quality and temperature gradients.

Dietary deficiency of essential polyunsaturated fatty acids is known to have greater impact on SGR at low temperatures (Masclaux et al. 2009), which is opposite to what we demonstrated here for P limitation. Since fatty acid and stoichiometric food quality most likely affect different mechanisms in organism SGR and reproduction, these effects could well operate independently, though with both being under the influence of temperature. We assumed that the P content was the only affecting food quality factor in our experiments; however, future work could ideally include a wider range of quality parameters to fully assess the relative importance of the various growth-promoting factors.

The elemental ratios in *D. magna* resemble those of other *Daphnia* species (Hessen 1990), which are generally known to show SGR retardation under P-deficient conditions (cf. Hessen et al. 2002). Hence, we believe the

observed temperature-stoichiometry in *D. magna* could be relevant in a broader context, both on spatial and temporal scales within ecosystems. Herbivores could thus run the highest risk of P limitation during summer while elemental ratios in food are expected to be less relevant in colder seasons, or in habitats with low temperature such as the hypolimnia of lakes. *Daphnia* that migrate to the colder metalimnion (e.g., McLaren 1963; Zaret and Suffern 1976) might be less sensitive to dietary P limitation in this region, while at the same time the available food in the metalimnion is expected to be of higher P content due to higher nutrient content and lower light intensity at these depths. One prediction would be that temperate species inhabiting cold lakes should be less negatively affected by dietary C:P compared to those living at higher temperatures, and also that the increased temperatures observed (and expected) in lakes worldwide (cf. Adrian et al. 2009) would enhance the risk of dietary P limitation. We believe this could be relevant not only for *Daphnia* spp. but for P-limited heterotrophs in general, and would thus represent a climate-induced check on productivity of such species. Adding to this, elevated levels of CO_2 may likewise induce elevated C:P in autotrophs, and thus increased risk of herbivore P limitation (Urabe et al. 2003). If herbivores additionally increase their requirement for dietary P in warmer temperatures, as the results from this study imply, the negative effects of food quality might get even more severe.

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