

# Joint effect of phosphorus limitation and temperature on alkaline phosphatase activity and somatic growth in *Daphnia magna*

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**Abstract** Alkaline phosphatase (AP) is a potential biomarker for phosphorus (P) limitation in zooplankton. However, knowledge about regulation of AP in this group is limited. In a laboratory acclimation experiment, we investigated changes in body AP concentration for *Daphnia magna* kept for 6 days at 10, 15, 20 and 25°C and fed algae with 10 different molar C:P ratios (95–660). In the same experiment, we also assessed somatic growth of the animals since phosphorus acquisition is linked to growth processes. Overall, non-linear but significant relationships of AP activity with C:P ratio were observed, but there was a stronger impact of temperature on AP activity than of P limitation. Animals from the lowest temperature treatment had higher normalized AP activity, which suggests the operation of biochemical temperature compensation mechanisms. Body AP activity increased by a factor of 1.67 for every 10°C decrease in temperature. These results demonstrate that temperature strongly influences AP

expression. Therefore, using AP as a P limitation marker in zooplankton needs to consider possible confounding effects of temperature. Both temperature and diet affected somatic growth. The temperature effect on somatic growth, expressed as the  $Q_{10}$  value, responded non-linearly with C:P, with  $Q_{10}$  ranging between 1.9 for lowest food C:P ratio and 1.4 for the most P-deficient food. The significant interaction between those two variables highlights the importance of studying temperature-dependent changes of growth responses to food quality.

**Keywords** Cladocerans · Compensation mechanisms ·  $EQ_{-10}$  · Growth ·  $Q_{10}$  value

## Introduction

A significant portion of body phosphorus (P) in fast-growing invertebrates is found in RNA (Elser et al. 2003). Owing to the generally tight coupling between [RNA], protein synthesis, and growth rate in many organisms (Sutcliffe 1970; Elser et al. 2003), dietary P availability can have a strong impact on the somatic growth rate and fitness of zooplankton (Sterner and Elser 2002). Therefore, efficient mobilization of phosphate from organic sources through catalysis by alkaline phosphatase (AP; EC 3.1.3.1) may be crucial for growth in an environment where P is limited. Several studies have shown AP to be indicative of bacterial or algal P status (Thingstad et al. 1998; Sebastian and Ammerman 2009). However, for zooplankton, this is not well studied. In zooplankton, AP activity has been demonstrated both within body tissues as well as in its excretion products (Jansson 1976; Boavida and Heath 1984). Indeed, AP has been partially purified from and characterized for crustaceans (Principato et al. 1984;

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Chuang and Shih 1990). Additionally, it has been shown that *Daphnia*'s AP has different biochemical properties than algal AP (Boavida and Heath 1984). In a biochemical study of AP excreted by *D. magna*, Zhao et al. (2006) presented detailed characteristics of the excreted enzyme and suggested a high relative stability with a potential for long-lasting catalytic activity. Recently, AP has been shown to be over-expressed in biomass of *D. magna* when raised on P-limited food (McCarthy et al. 2010), which may imply a direct role of animal-AP in P acquisition. In another study, protein-normalized AP in individual *D. magna* was constant over the first 6 days of development when animals were constantly fed P-rich, low C:P food (Wojewodzic et al., in review).

Temperature is a central regulator of metabolism because it directly influences all physiological and biochemical functions (Hochachka and Somero 1973). Growth, element acquisition, and enzymatic activity are all highly dependent on temperature. However, poikilothermic animals actively adjust after an acute drop in environmental temperature by physiological modifications over a period of acclimation (Nathanailides 1996). One of the prime responses to low temperature is to compensate for reduced catalytic rates of enzymes at low temperatures. At least two types of adaptations can promote high activity of enzymes at low temperatures (Hochachka and Somero 1973; Somero 1995). First, a higher concentration of intracellular enzymes, resulting in an increased number of catalytic sites, can compensate for the lower temperature-induced rate per site. Secondly, a higher inherent catalytic activity per active site can be induced, such that fewer molecules are needed to maintain the same catalytic rate (Somero 2004). Both over-expression of enzymes at lower temperatures and changes of enzyme substrate affinity have been reported in seasonal acclimation studies (Hazel and Prosser 1974; Sidell 1977). The biochemical mechanisms behind low temperature adaptation may also involve adding low-molecular-mass constituents or making covalent modifications to the protein (Somero 2004). Enzymatic temperature compensation has been evaluated in numerous organisms. One of the most studied examples is the temperature adaptation of lactate dehydrogenase orthologs isolated from vertebrates, where increased enzyme efficiency at low temperatures results from an increase in the catalytic constant ( $k_{cat}$ ) (Fields and Somero 1998; Somero 2004). In contrast to these evolutionary adaptations, compensation mechanisms during physiological acclimatization are limited to changes in enzyme expression, mainly expression of the same isoform or by producing a new isoforms with different kinetic properties.

The temperature effect on biological process rates is often described by a  $Q_{10}$  parameter that represents the relative change in the process rate for a 10°C temperature

increase. The  $Q_{10}$  itself will not be temperature-independent unless the process rate scales exponentially with temperature. Process rates  $k_1$  and  $k_2$  at temperatures  $T_1$  and  $T_2$  will be related through the  $Q_{10}$  by the following relationship:

$$\frac{k_2}{k_1} = Q_{10}^{\frac{T_2-T_1}{10}} \Leftrightarrow Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10}{T_2-T_1}} \Leftrightarrow Q_{10} = \frac{\left(\frac{k_1}{k_2}\right)^{10}}{t_1 - t_2} \quad (1)$$

A value of  $Q_{10} > 1$  means that the biological process increases with temperature, while a value below 1 suggests a decrease or even potential damage by irreversible loss of function. Typical  $Q_{10}$  values for biological systems are often found to be around 2, especially when the  $Q_{10}$  is studied close to physiological optimum temperatures. *D. magna*, used in our study, tolerates temperatures between 2 and 30°C (Goss and Bunting 1983), while the optimum temperature range for assimilation, growth, and reproduction has been shown to be in the range 15–25°C (Lampert 1977; Kersting 1978; Goss and Bunting 1983). At the same time, excreted AP from this species has been found to be stable at 13–65°C, with maximum activity between 30 and 35°C (Zhao et al. 2006). The same study revealed that the activity of *Daphnia* AP increased exponentially with temperature below 30°C but decreased above 35°C, which does not match the optimal temperature for somatic growth of this species. Zhao et al. (2006) determined the  $Q_{10}$  for excreted AP activity to be 2.45. Changes in body concentrations of an enzyme can be expressed as an  $EQ_{-10}$  value, which characterizes how much the activity of enzyme ( $E$ ) increases with every 10°C drop in temperature acclimation:

$$EQ_{-10} = \left(\frac{V_2}{V_1}\right)^{\frac{-10}{T_2-T_1}} \quad (2)$$

where  $T_1$  and  $T_2$  represent acclimation temperatures (°C) and  $V_1$  and  $V_2$  are units of enzyme activity measured at the same temperature. The  $EQ_{-10}$  parameter has never been determined for AP from *Daphnia*. The ratio of  $EQ_{-10} - Q_{10}$  for an enzyme expresses its compensation efficiency. A value of compensation efficiency close to 1 suggests perfect compensation in the system, while a quotient lower than 1 suggests imperfect compensation (Nathanailides 1996).

Given the major impacts of food quality and temperature on consumer growth rate, it is important to understand how animals cope with P deficiency under various temperatures. Here, we have addressed this by studying AP activity in the key pelagic grazer *Daphnia* along gradients of dietary C:P and temperature. However, given the likelihood of P-limitation in other organisms, including terrestrial invertebrates (Perkins et al. 2004), we believe these findings should have relevance outside the aquatic realm. We also

assess the use of AP to study animal P limitation in situ as an alternative to more traditional and laborious experimental approaches (e.g., P enrichment experiments, such as Elser et al. 2001). Since animal populations in situ experience different temperatures, the effects of temperature on AP activity should also be considered in such studies.

## Materials and methods

The green alga, *Selenastrum capricornutum* Printz (Norwegian Institute of Water Research, Oslo) was grown in continuous culture (dilution rate  $0.2 \text{ day}^{-1}$ ) in 2-L glass vessels. Light levels were nominally at  $70 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  using 25-W blue-white fluorescent tubes, and the temperature was  $20^\circ\text{C}$ . Sterile media used for growing algae was either complete COMBO with  $50 \mu\text{M}$  P as orthophosphate, or P-limited COMBO with only  $2 \mu\text{M}$  P and the remainder replaced with an equivalent amount of KCl (Kilham et al. 1998). Cultures were used when they reached a stable rate determined by the culture optical density (OD) at 633 nm. Algal biomass was collected on previously pre-combusted (3 h,  $500^\circ\text{C}$ ) GF/F filters (Whatman, Kent, England) for further determination of particulate C and P content. Filters were dried for carbon analysis or frozen and kept at  $-20^\circ\text{C}$  for further P analysis. Algal C and N content was measured on a Flash EA<sup>TM</sup> 1,112 automatic elemental analyzer (Thermo Finnegan, Milan, Italy) and total P content was measured by persulfate digestion followed by a molybdate-blue assay (Menzel and Corwin 1965). A standard reference material (apple leaves, material 1515; National Institute of Standards and Technology, USA) was used to confirm the accuracy of analytical methods for both P and C.

To create a gradient of algal C:P ratio for feeding to animals, P-rich cultures (A) were mixed with P-limited cultures (J) in varying proportions for obtaining the desired food quality, yielding molar C:P ratios of 95 (A), 110 (B), 169 (C), 228 (D), 318 (E), 401 (F), 466 (G), 509 (H), 563 (I) and 663 (J). To make the daily treatments, algal cultures were mixed, concentrated using  $0.45\text{-}\mu\text{m}$  millipore filters, washed and re-suspended in 50 mL of N and P free COMBO at a final concentration of  $3 \text{ mg C L}^{-1}$ . The C content was estimated daily from previously established regression line between OD and C content. Food mixtures were sampled daily for analysis to assure that the C:P range was stable over the duration of the experiment.

Stock cultures of *D. magna* Straus were kept in the laboratory for >6 months at  $20^\circ\text{C}$  in N- and P-free COMBO medium and fed P-sufficient algae ad libitum. Density of cladocerans in the cultures was always kept below stress conditions. All glassware was soda and acid washed, rinsed with distilled water, and autoclaved prior to

use to avoid bacterial and phosphate contamination. Animals used in experiments were all from the second brood of their mothers and less than 15 h old.

Feeding experiments were conducted to obtain estimates of food residence time, defined here as the time necessary for animals to replace their gut contents. Since *Daphnia* does not discriminate between individual particles on a chemosensory basis (DeMott 1986), and filtering efficiency of *D. magna* is independent of the particle size (Lampert 1987), fluorescent beads were used in three related feeding experiments. First, saturation time was determined by microscopic observations of animals feeding on  $6\text{-}\mu\text{m}$  fluorescent beads with concentration  $8.7 \times 10^7$  particles  $\text{L}^{-1}$  (17156, Fluoresbrite YG Microspheres; Polysciences, USA) at 5-min intervals for 30 min. Knowing the saturation time, a second experiment was designed to monitor evacuation rate. Seven-day-old *D. magna* were fed fluorescent beads for 10 min, washed in COMBO, and transferred to medium containing algae ( $3 \text{ mg C L}^{-1}$ ). Six animals at a time were then collected after 0, 10, 20, 40, 60, and 120 min of grazing and washed twice in COMBO medium followed by sonification in  $300 \mu\text{l}$  of new COMBO. Subsamples ( $200 \mu\text{L}$ ) were transferred to a 96-well plate (655076; Greiner Bio-One, USA) and the fluorescent signal was measured at an excitation wavelength of 460/40 nm and an emission wavelength of 528/20 nm using BioTek FLx800 plate reader (BioTek, USA). In a final experiment under similar conditions, animals were collected at 0, 1, 5, 10, 15, 60, and 120 min and inspected microscopically for any remaining fluorescent signal in the gut (Axio Scope A1 microscope; Zeiss).

Individuals of *D. magna*, <15 h old, were randomly distributed to 80 sterile 50-mL glass bottles containing algal suspensions with 10 different C:P food levels, all at  $3 \text{ mg C L}^{-1}$ . Bottles were held at 10, 15, 20 and  $25^\circ\text{C}$  in temperature-controlled incubation rooms with a light:dark cycle of 16:8 h. Animals were gently transferred daily by pipette into freshly made food while maintaining treatment temperatures. The temperature of each climate-controlled room was monitored continuously by a data logger (Log-Tag Recorders, New Zealand). For each treatment (10 different diets  $\times$  4 temperatures), there were two replications and four individuals within each replicate. To avoid pseudo-replication, we averaged measurements on the four individuals to get a single value for each replicate bottle (Sokal and Rohlf 2010).

Animals used in the experiment were kept on the same type of food and the same temperature before entering the actual experiment; therefore, any observed differences must be classified as acclimation effects.

To normalize the gut content prior to analysis, individual animals were removed from their experimental treatments after 6 days, and placed into sterile containers with

10 mL of P-rich algae at  $3 \text{ mg C L}^{-1}$ . They were then allowed to graze for 40 min in order to replace the gut contents with P-rich (low AP) algal food. After this treatment, single animals were washed in P-free COMBO, photographed (Nikon D-50) for body weight estimation, placed individually into pyrophosphate-free microcentrifuge tubes (MCT-150-C; Axygen, USA), and snap-frozen in liquid nitrogen. Animals were stored at  $-80^\circ\text{C}$  and analyzed within the same week.

Individuals were analyzed for AP activity using the CDP-Star chemiluminescence method (Wojewodzic et al., in review). AP activity was normalized by total protein measured directly in the same extract from a single homogenized individual. Briefly, AP from animals was extracted by 2-min sonification in a cuphorn (Brandson 101147048) with 1% Triton X-100 (Sigma-Aldrich, 93443, hereafter called Triton), and chemiluminescent signal measured in plate reader at ambient room temperature (FLx800; BioTek) after addition of ready-made CDP-Star substrate (0.4 mM, T2214; Applied Biosciences, USA). Bovine AP (Sigma-Aldrich, P5521) was used for calibrating the luminescence signal to enzyme activity units. Traditionally one unit (U) is defined as the amount of enzyme required to hydrolyze  $1 \mu\text{mol}$  of 4-nitrophenyl phosphate per min at pH 9.8 at  $37^\circ\text{C}$ . Protein content in the residual extract was quantified according to bicinchoninic acid method (Smith et al. 1985) by a commercial kit (Pierce, 23225). Low range standards for this quantification were prepared from bovine serum albumin supplied by the same manufacturer. Protein samples were incubated for 30 min at  $37^\circ\text{C}$  before measuring OD at 562 nm using Synergy 4 plate reader (BioTek). Dry weights of the animals were estimated from body length measured from images taken before snap-freezing, converted to dry weight from a previously determined regression relationship.

AP activity in animals from all experimental treatments was measured at standardized conditions (identical gut purging procedure, same AP assay incubation temperature). Since animals raised at different treatment temperatures were extracted and assayed for enzymatic activity at the same incubation temperature ( $20^\circ\text{C}$ ), the temperature response of these measurements would correspond to an  $EQ_{-10}$  as defined by Nathanailides (1996). To correct AP activities measured under the same assay incubation temperature to the actual enzyme activity at the treatment temperature, the  $Q_{10}$  parameter previously determined by Zhao et al. (2006) was applied. Finally, the  $EQ_{-10}:Q_{10}$ -ratio was calculated to assess the temperature compensation efficiency of the AP enzyme (Nathanailides 1996).

Both somatic growth and AP activity were analyzed by general linear models using diet, temperature, and their interaction as explanatory variables. The necessity of including the interaction components into models was

checked by extra sum of squares tests. We also inspected residual plots to assess if the models fulfilled the assumptions for linear regression (linearity, normality, and homoscedacity). Data were log-transformed to account for the scale effects that could possibly occur in this experimental approach (Stanton and Thiede 2005; Stillwell et al. 2007). Statistics were carried out using either R software (R development core team, Vienna, Austria) or Graph Pad Prism 5.00 (Graphpad Software, San Diego, USA).

## Results

The chemostats used for this study were at steady state as indicated by stable daily readings of  $OD_{633}$  (data not shown). Subsets of the 10 different food treatments were sampled and analyzed for C and P content following daily preparation. Internal controls (apple leaves) included for P and C analysis resulted in 95, 97 and 96% recovery for P, C and N, respectively. Animals in the different treatments received a constant carbon concentration of  $3 \text{ mg C L}^{-1}$  ( $\pm 0.3$ ) while the C:P and C:N ratio differed across food quality treatments and was stable within treatments during the 6-day experiment (Table 1).

The gut-clearance assessment using animals fed fluorescent beads for 30 min showed that a 10-min feeding period was sufficient for the animals to fill their guts with fluorescent beads (data not shown). Accordingly, we let another set of animals feed on beads for 10 min before washing in COMBO and transferring them to new vials with  $3 \text{ mg C L}^{-1}$  of algae. The remaining bead fluorescence was then measured at different time intervals in a plate reader after homogenization. Animals replaced their gut contents relatively quickly, showing a short decay time

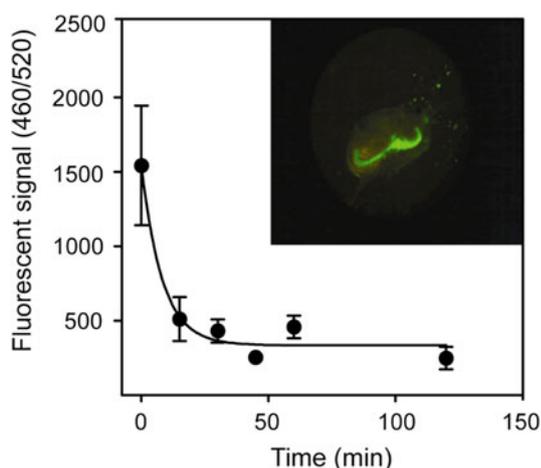
**Table 1** Food treatments used for the 6-day feeding experiment of *Daphnia magna*

Diet	Average C:P	SD	Average C:N	SD	n
A	95	2	7.4	0.4	6
B	111	20	8.9	0.8	6
C	167	17	10.1	1.2	6
D	228	16	11.5	0.9	6
E	319	16	11.8	2.1	6
F	401	29	12.0	0.7	6
G	466	31	12.7	1.8	6
H	509	46	12.8	1.9	6
I	563	77	12.3	0.9	6
J	663	68	13.3	1.2	6

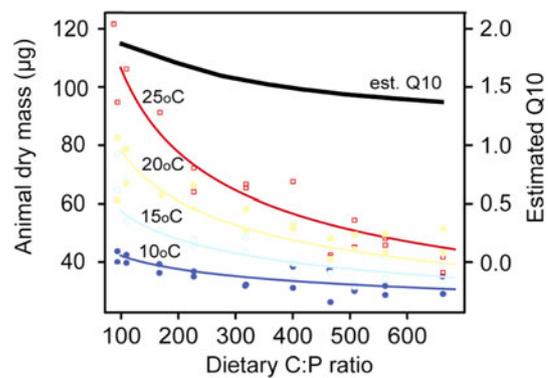
A gradient of C:P:N ratios (molar) was obtained by mixing chemostat grown P-limited and P-rich cultures of *Selenastrum*. Samples were collected daily over the 6-day period

(Fig. 1). However, it was difficult to determine the exact time when no residual beads were present in the gut. This is probably due to adhesion of the beads to both the external carapace and the antennae of cladocerans (Fig. 1). An additional experiment was then conducted in which animals were visually inspected by epifluorescence microscopy. Here, we found that an incubation time of 40 min was optimal for replacing the gut contents by new algal food (data not shown). Thus, by feeding *Daphnia* on P-rich food for 40 min prior to collection, we can eliminate potential bias due to differences in AP activity of different food treatments, as the AP activity contributed by P-rich algae in the gut has been found to not interfere with measurements of animal body AP (Wojewodzic et al., in review).

No mortality was observed during the course of the experiments. Log-transformed body weights on day 6 of the experiment were fit to a linear model with temperature, log-transformed food C:P ratio, and their interaction as independent variables. This model explained 86% of the variance in the observed dry weights of the animals and was highly significant ( $F_{3,73} = 150.1$ ,  $P \ll 0.001$ ). An extra sums of squares ANOVA test also revealed a significant interaction between the two treatment factors ( $F_{1,73} = 23.7$ ,  $P < 0.001$ ). Temperature explained 46.5% of the observed variation while 35% of this variation was explained by diet and only 4.5% by the interaction. Owing to the interaction effect, the calculated  $Q_{10}$  parameter for somatic growth was dependent on the C:P ratio of the diet (Fig. 2).  $Q_{10}$  values for growth decreased from 1.8 when animals were fed a P-rich diet to 1.4 when P-limited food was offered. Based on differences in body masses,



**Fig. 1** Time course of gut content replacement for *Daphnia magna*. Fluorescent signal is residual bead fluorescence in algae-feeding animals that have been grazing 10 min on fluorescent beads (mean  $\pm$  SD,  $n = 6$ ). Right corner inset *Daphnia magna* with the gut filled with fluorescence beads after 10-min grazing period

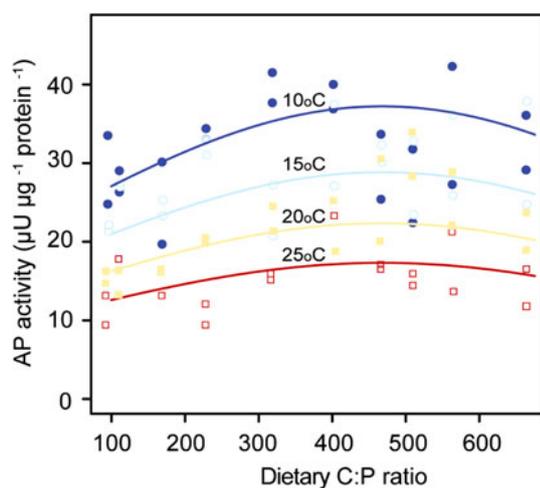


**Fig. 2** Joint effect of dietary C:P ratio (molar) and temperature (10, 15, 20, and 25°C) on body dry mass of 6-day-old *Daphnia magna*. Each data point represents the mean of one replicate, the average of four animals. The model equation was  $\log_{10}(\text{dry mass}) - 3.01 + (0.151 \times \text{temperature}) + 0.0259(1 - 0.75 \times \text{temperature}) \times \log(\text{C:P ratio})$ . Estimated  $Q_{10}$  parameter was calculated for somatic growth of *Daphnia magna* (right hand y-axis; heavy solid line est.  $Q_{10}$ )

*D. magna* was severely P-limited at 25°C while only modest P limitation was observed at 10°C.

Measurements of AP activity were done at the same temperature (20°C), which enabled a comparison of the temperature treatments (Fig. 3). A linear model was used to describe the joint effect of both temperature and dietary C:P ratio on the protein-normalized AP activity in acclimated animals. The AP activity measurements were log-transformed to ensure normality and homoscedasticity of the residuals. A quadratic term involving diet C:P ratio was needed to remove non-linearity in the residual plots. After simplification by the Bayesian Information Criterion (BIC; Schwarz 1978), the model explained 70% of the observed variation in the data. An extra sum of squares test showed that the interaction term was not significant ( $F_{2,71} = 0.97$ ,  $P = 0.38$ ) and therefore was not incorporated into the model. There were strong effects of both temperature and diet (overall  $F_{3,73} = 57.9$ ,  $P \ll 0.001$ ) on protein-normalized enzyme activity. Additionally, the temperature effect on AP activity was found to be much stronger, explaining 61% observed variation compared to the diet treatments, which explained 9% of the variation (Fig. 3). For all temperatures, the somatic AP activity increased when animals were fed with low-P food; however, a striking decrease of the AP activity was observed when C:P ratio in the offered food exceeded 500.

The fitted model had log-transformed AP activity and no interaction terms with temperature, implying that, if the diet C:P ratio is kept constant, AP activity is an exponential function of temperature. This also means that the  $EQ_{-10}$  for AP activity is constant and independent of dietary C:P ratio. Moreover, the  $EQ_{-10}$  can be estimated from the regression coefficient for temperature ( $-0.051$ ) as



**Fig. 3** Joint effect of C:P food ratio (molar) and temperature (10, 15, 20, and 25°C) on body AP activity of six-day-old *Daphnia magna*. Each data point represents the mean of one replicate, the average of four animals. The model equation was:  $\log_{10}(\text{AP activity}) = 3.62 - (0.0510 \times \text{temperature}) + 0.00216 \times \text{C:P ratio} \times [0.00000228(\text{C:P ratio})^2]^{-1}$  AP activity was assayed at 20°C for all temperature and diet treatments

$EQ_{-10} = \exp[-10(-0.051)] = 1.67$ , which implies that activity increased by a factor of 1.67 for every 10°C decrease in temperature.

Finally, to assess the compensation efficiency, the  $EQ_{-10}:Q_{10}$ -ratio was calculated. We used the  $Q_{10}$  value reported by Zhao et al. (2006) for AP excreted by *D. magna*. In our study, cold acclimation resulted in achieving 68% compensation, classifying it, after Nathanalides (1996), as an imperfect compensation.

## Discussion

The study design yielded a wide gradient in dietary C:P-ratios. The P-deficient algae also had somewhat lower N-content than the P-sufficient algae, probably due to somewhat reduced protein synthesis under P-deficiency. Over this range of C:P and C:N ratios, it is very unlikely, based on Hessen et al. (2002), that the threshold elemental ratio with regard to N should be exceeded for *Daphnia*. We also believe that, if any effects of slight nitrogen changes in the diet on AP expression occurred, they were counteracted by normalization of body AP activity against body protein content.

A critical step in performing these experiments was to ensure negligible AP contribution from the gut contents of the experimental animals. Since starvation does not promote gut evacuation in zooplankton (Gillis et al. 2005), it was necessary to actively purge the gut with low AP food to ensure uniform gut content across treatments. The feeding and evacuation experiments with fluorescent beads

increased confidence that the chosen experimental protocol achieved this goal. P-sufficient and AP-suppressed algae have been shown not to contribute to the AP signal of the grazer and have been successfully used to eliminate possible interference from foreign AP sources (Wojewodzic et al., in review). This, in combination with the rate measurements, gave us a more precise method for eliminating the interference from the gut content treatments and a rapid exchange of food that would limit any transient effects of the food on expression of the AP by the animal. We realize that such P-rich food might potentially inhibit the animal's AP activity; however, McCarthy et al. (2010) performed P-spiking experiments on *Daphnia* extracts without apparent significant inhibition of enzyme activity. In addition, P-rich algae are not necessarily rich in orthophosphate. Therefore, the combination of short grazing times with AP-suppressed algae is an effective means to create a uniform gut content across treatments.

Prior to starting these experiments, we considered use of dietary antibiotics to minimize the impact of bacteria but did not employ them because antibiotics may potentially alter animal AP expression. Instead, we used sterile media, bottles, pipettes and chemostats with sterile COMBO medium. We believe these precautions substantially decreased bacteria growth. We also conclude that bacterial impacts are unlikely given our observation of high AP quantity per individual found at low temperatures compared to animals from higher temperatures—the pattern opposite to what one would expect if bacterial contributions were important.

We used the mild detergent Triton to release AP bound in membranes. Previous histological methods have demonstrated AP activity in the midgut of the copepod *Centropages typicus* (Arnaud et al. 1984). High AP activity has also been found in shrimp hepatopancreas, a gland tightly associated with digestive systems (Principato 1984; de-Backer et al. 2002), suggesting a direct link between enzyme production and excretion into the digestive system. In the decapod, *Penaeus japonica*, AP has been shown to be anchored by a phosphatidylinositol-glycan into the cell membrane (Chuang and Shih 1990). Interestingly, the genome of *D. pulex* (a close relative of *D. magna*) suggests the presence of at least three genes coding for membrane-anchored isoenzymes of AP (McCarthy et al. 2010). Given these findings, it is likely that AP is found in cell membranes of zooplankton and that Triton is a reasonable choice for a detergent to dissolve the membranes and release AP while maintaining the enzyme activity (Boavida and Health 1984).

We measured somatic growth of the animals acclimated for 6 days to the broad range of low-P food and four temperatures. Consistent with previous work (Urabe et al. 1997; Brett et al. 2000; Hessen et al. 2002), the somatic

growth rate of cladocerans decreased as the C:P ratios in the diets increased. In our study, this P-limitation effect was most pronounced at 25°C, suggesting that anabolic processes are more P-demanding when animals grow at higher temperatures. On the other hand, the animals kept at 10°C showed only weak signs of P-limitation, suggesting that temperature ameliorates nutritional limitations at low temperatures. Our findings are in agreement with another growth rate study where *Daphnia* were raised under different temperatures and across C:P ratios, but where the food treatments were created by spiking P-limited algae with different amounts of inorganic P (Persson et al., in review). An increase of temperature promotes somatic growth of *Daphnia* under three conditions: (1) growth occurs in the physiological optimum temperature range for this species; (2) the food has sufficient P content for the growth requirements; and (3) sufficient food is available. A possible ecological relevance of this finding is that food quality effects may thus co-vary with temperature scales within any given lake—or between lakes. The somatic growth of animals kept on poor quality (low C:P) food will be more affected by the same increase in temperature than those growing on high quality (high C:P) food. This also means that increasing temperature can result in further escalation of P limitation for herbivorous cladocerans. The significant interaction between temperature and food quality makes the  $Q_{10}$  parameter for somatic growth rate dependent on C:P ratio. This again points out that the somatic growth of cladocerans is being shaped by temperature, but in a food quality-dependent manner. Ecological consequences of this interaction could appear along temporal (diurnal cycles, seasons) and spatial (depth, altitude, latitude) temperature gradients. For example, the diel vertical migration of zooplankton, considered one of the largest animal biomass movements relative to body size (Hays 2003), can easily occur across gradients of 15°C. Additionally, the seston composition is often not homogeneously distributed, either quantitatively (Dagg 1977; Dagg et al. 1997) or qualitatively (Williamson et al. 1996), in stratified lakes. Such combinations of factors may result in interactive changes for animal growth dynamics and have further consequences for animal fitness and resource acquisition strategies. Field studies involving AP may have to carefully consider the varying temperature and food regimes animals experience during the course of their daily movements.

While temperature also had the expected positive effect on growth in our experiments, we found the opposite effect on protein-specific AP activity, which actually decreased with increasing temperature. Since all experimental treatments were terminated the same day, animals had different sizes depending on treatment. Owing to the observed C:P ratio and temperature effects on AP, the changes found

could potentially be indirect effects of size if AP activity somehow relates to ontogeny. As much as 86% of the observed variance in dry weight could be explained by the C:P ratio in the diet and temperature while only 70% of the variance in AP activity normalized by protein was explained with the same variables. However, since we can explain only 62% of the AP activity variance by animal dry weight (data not presented) and temperature (i.e., less than using C:P ratio and temperature), we infer that the small, but significant, C:P ratio effect on AP normalized by protein is not just animal size in disguise but a real effect of P limitation. The fact that *D. magna* kept on a P-rich diet during the first 6 days had a constant protein-specific AP activity (Wojewodzic et al., in review) also supports the view that the treatment effects that we see are not ontogeny related.

Our results align well with changes in AP expression within the body of *D. magna* recently demonstrated as a response to nutritional P limitation by McCarthy et al. (2010), providing further support the hypothesis that AP activity increases in P-limited *Daphnia* as a potential mechanism for increasing P sequestration from ingested food. McCarthy and colleagues kept *Daphnia* on P-limited diet and measured AP activity within the body of animals without correcting for differences in the AP activity of the gut contents. While their AP expression patterns were similar to what we report here, McCarthy et al. (2010) did not find any decline in the AP activity at high dietary C:P ratios. This difference could be explained if the data collected from the highest C:P treatments in the McCarthy et al. (2010) study were biased by highly over-expressed AP from P-limited algae in the animal's guts.

Animals experiencing extreme P limitation exhibit decreases in RNA production and somatic growth along with other physiological malfunctions (Brett et al. 2000; Elser et al. 2001; Hessen et al. 2002; Vrede et al. 2002; Seidendorf et al. 2009). In our study, the down-regulation of AP activity within the body of highly P-limited animals might be due to a decrease in RNA concentrations followed by a general decrease in the efficiency of transcription processes, consequently causing not only a smaller body size phenotype but also impairment of protein synthesis, including the AP enzyme. An alternative explanation for this decline in AP-expression is the possible existence of a trade-off between production of the constitutive proteins (related to somatic growth) and expression of AP in a body already facing growth difficulties. McCarthy et al. (2010) suggested the presence of a trade-off between excreted and membrane-bound AP as a response to increased dietary C:P ratio. They suggested that animals over-express AP within their body while decreasing excreted AP. This was hypothesized as a shift in expression of different AP isoforms from excreted fractions to membrane-anchored

forms. Since we did not focus on the measurement of excreted AP, we cannot test this hypothesis.

This is the first time that AP has been studied in the context of P-limitation and temperature simultaneously. AP activity increased with decreasing temperature. Furthermore, temperature effects on AP were much stronger than P effects. We propose that higher AP activity found at low temperature treatments is a compensatory mechanism against decreased enzyme activity at low temperature, a consequence of the Arrhenius law for reaction kinetics. Such a compensation effect was proposed by Somero (2004), and demonstrated for other enzymes (Fudge et al. 1997), and further discussed in the context of ecological stoichiometry (Woods et al. 2003). The estimated  $EQ_{-10}$  coefficient for *D. magna* AP expression of 1.67 suggests that AP activity increases relatively quickly when temperature decreases. The enzyme temperature stability range of AP (10–60°C, Zhao et al. 2006) excludes the possibility that observed differences were due to loss of function at higher temperature but rather suggests a scenario in which the enzyme molecules are accumulated (over-expressed) as a response to lower temperature. The estimated  $EQ_{-10}$  coefficient for AP expression could be used if comparison of AP data from different temperature sites is desired. The efficiency of this compensation (68%) suggests an imperfect compensation process is taking place (where cold acclimation does not influence the magnitude of the response exhibited), possibly allowing other mechanisms to modulate AP function at lower temperature.

The increased animal AP activity at lower temperatures might be due to an overall increase of enzyme molecule number appearing during the 6-day acclimation of cladocerans to low temperature treatments. When measured under optimal assay conditions, maximal activities of enzymes are thought to accurately reflect the quantity of enzyme molecules present (Sidell 1983). However, many genes coding for AP homologs are known in the genome of *D. pulex*, and the active products of these genes, different AP enzymes, can potentially vary in biochemical characteristics. Therefore, we cannot rule out the possibility that different AP isoenzymes are expressed at different temperatures and that these may also have slightly different biochemical properties, such as half-saturation ( $K_m$ ) and asymptotic rate ( $V_{max}$ ) parameters of the Michaelis–Menten model. Further purification, separation (such as 2D gel analysis), and kinetic characterization ( $K_m$  and  $V_{max}$ ) performed on extracts from different temperature treatments might answer this question.

Another alternative mechanism for the observed modulation of AP activity is via changes in the membrane milieu where proteins function. One way of achieving this is through changes in the membrane lipid composition, which have been demonstrated previously for *Daphnia* in response

to low temperature (Chapelle 1978; Farkas 1979; Brenner 1984; Pruitt 1990). Such a mechanism of modulation would not be detected by our AP quantification method since cell membranes were dissolved during the AP extraction. However, the possibility remains that the mechanisms involved in changing AP activity in *D. magna* acclimated to different temperatures and diet involved not only an increased number of transporters and different expression patterns of iso-enzymes but also protein function modulation. This could possibly explain the occurrence of imperfect compensation we observed for *D. magna* AP.

Phosphorus acquisition is an essential process for ensuring satisfactory growth and thus maximizing fitness of an organism. The expression of AP may serve as a crucial tool for acquiring this nutrient under P-limited conditions. Our study demonstrates that quantification of body AP can be successfully used to assess the status of metazoan P limitation in situ in the moderate range of C:P ratio ( $\leq 500$ ) which still represents the broad working range of C:P found in freshwater systems. However, as we show here, serious difficulties in data interpretation may occur if seston C:P ratio surpasses the presented limits of the method, at least for *D. magna*, or the temperature effects on AP enzyme expression are not assessed properly. With these caveats in mind, the presented technique has several advantages relative to more conventional time-consuming experimental approaches, and could be applicable for a wide range of metazoans in various habitats. This will allow a more widespread assessment of the operation of dietary P-limitation of animals under in situ conditions. In fact, despite these difficulties, Elser et al. (2010) were, after temperature correction of AP activity, able to successfully document increased animal P limitation associated with elevated seston C:P ratios in Norwegian lakes receiving elevated atmospheric N deposition. Given the likelihood of P-limitation for other grazers feeding on P-deprived plant matter, including terrestrial invertebrates (Perkins et al. 2004), this method could be relevant in a wide range of organisms.

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