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Stoichiometric regulation of carbon and phosphorus in P-deficient *Daphnia magna*

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Abstract

Daphnia magna were fed phosphorus-sufficient (+P) and P-deficient (−P) green algae *Chlamydomonas reinhardtii* (carbon : phosphorus ratio of C : P = 90 and 930 in molar, respectively) for 5 d to produce different body C : P ratios. The dietary absorption as well as the elimination of body C and P were then quantified under contrasting dietary qualities (+P and −P). The −P animals fed with −P algae had a higher absorption efficiency (AE) of both C (46%) and P (52%) than the control (+P animals fed +P algae) and the recovery group (−P animals fed +P food). During the physiological efflux, the −P animals fed with −P diet eliminated their body C at the highest rate (0.41 d^{-1}) and their body P at the lowest rate (0.10 d^{-1}) among the three groups of animals. Mass-specific C loss rates through dissolved release, respiration, and molting increased significantly, and the mass-specific P loss through dissolved release, molting, and reproduction decreased in the −P animals compared with the +P animals, in agreement with the stoichiometric models. Consequently, the C : P ratio of dissolved release, molting, and reproduction all increased with the increase in P deficiency. The recovered *Daphnia* had medium values of AE, efflux rate constant, and mass-specific loss rates, indicating the reversibility of P limitation. Our study demonstrated that all the pathways (excretion, reproduction, molting, and respiration) may be involved in the stoichiometric regulation in *Daphnia*.

The phosphorus (P) content of algal cells as a component of food quality directly affects the growth and reproduction of freshwater zooplankton and eventually the zooplankton community structure in lake systems (Hessen 1992; Sterner and Hessen 1994; Hassett et al. 1997). Freshwater zooplankton *Daphnia* have a P content of 1–2% of dry weight, which is higher than other crustacean zooplankton; thus, compared to other cladocerans, *Daphnia* are likely more often limited by P. Compared with the rather variable carbon : phosphorus (C : P) ratios in phytoplankton, *Daphnia* keep their stoichiometry more constant, although their C : P ratios may also vary slightly under some circumstances (Hessen and Lyche 1991; DeMott et al. 1998; Sterner and Schulz 1998). The cladocerans undergo ontogenic changes in their elemental compositions, and generally the faster-growing juveniles have higher P content than the slower-growing adults (Baudouin and Scoppa 1975; Main et al. 1997; Sterner and Schulz 1998). DeMott et al. (1998) and DeMott (2003) demonstrated that the P-deficient diets led to significant declines in *Daphnia*'s P content (e.g., the specific P content decreased from 1.5% to 1.1% over the range of dietary C : P ratios of 80–900). These studies imply that the P content of zooplankton may be closely linked to the P content of their diets and may vary under P limitation. It is well known that the sestonic C : P ratios in lakes vary seasonally from P sufficiency to P limitation (Anderson and Hessen 1991; Ventura and Catalan 2005). According to a simple kinetic model, the transfer of elements along a food chain is controlled by the dietary absorption efficiency, efflux rate, and ingestion activity (Wang 2002). Thus, it is important to understand the control of each kinetic parameter in the body stoichiometry of elements in zooplankton. These parameters may provide helpful information to understand the stoichiometric regulation in animals.

Ingestion activity is a function of nutritional provision, including food quantity and quality. Several studies have addressed the implications of nutrient limitation on zooplankton, but controversial results have been obtained on the feeding responses (ingestion and filtration rates; DeMott et al. 1998; Plath and Boersma 2001; Darchambeau et al. 2003). Under P limitation, the C growth efficiency (production : ingestion) decreased, whereas the P growth efficiency exhibited a unimodal pattern (DeMott et al. 1998). Furthermore, because of the potential trade-off in the allocation of C or P to somatic or reproductive tissues, P limitation may constrain reproduction in the way different from the shortage of energy (carbon) (Færøvig and Hessen 2003). In this circumstance, the change in the allocation of C and P in reproduction in mature individuals might be expected. Another important process, namely, molting, is also a considerable drain on P (e.g., up to 15% of body P is carapace bound in *Daphnia*; Vrede et al. 1999) and C (about 2.5% of body C was lost through molting per day; Lampert and Bohrer 1984). These processes need to be considered during the efflux process as far as the responses of crustaceans to the shift in nutrient condition are concerned. Even though some stoichiometric models have considered the growth and molting (e.g., Anderson et al. 2005), so far there has been no direct experimental effort to integrate the allocation of energy (carbon) and elements in growth, molting, and reproduction when the animals are challenged with food of low quality.

To maintain a relatively tight homeostasis, there must be some strategies of stoichiometric regulation in zooplankton

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to deal with the inferior nutrient conditions. Some potential physiological strategies are used by the homeostatic zooplankton coping with less P and excess C in the diets when the food quality cannot match the demand of the animals. These include (1) improvement of the P absorption but decrease of the C absorption, (2) storage of assimilated C internally in the C-rich compounds, and (3) disposal of assimilated excessive C through respiration and excretion. DeMott et al. (1998) showed that an increased C : P ratio in the diets caused a decrease of C absorption, while the P absorption was not affected by the P availability in food. The second strategy, namely, the C storage, may not be applicable for animals to control the body C : P ratio. Darchambeau et al. (2003) reported that *Daphnia* on high C : P diets had higher respiration and excretion rates in terms of C than those on low C : P diets. A recent model of the stoichiometry of consumers suggested that the release of excess elements (in organic form) in the food may also be one mechanism in elemental stoichiometric regulation while the assimilation efficiency is maintained constant (Anderson et al. 2005). Urabe and Sterner (2001) proposed another mechanism, the production of nonviable eggs, which could not be fully developed because of low P content. Reproduction may cause temporal change in zooplankton body stoichiometry (Ventura and Catalan 2005). To our knowledge, there is no study on P limitation in crustaceans that integrates all the relevant processes. Such information is nevertheless critical for our understanding not only of the elemental homeostatic regulation in zooplankton but also of the eventual consequences brought by the elemental limitation to associated processes in the environment.

In this study, a radioactive dual-tracer technique was used to examine the kinetics of P and C in *Daphnia magna* under P-sufficient and P-deficient preconditioning. We specifically addressed the influences of feeding history on the kinetics of P and C in the animals. It is well known that when the C : P ratio is well above 300 in molar, mineral limitation plays a major role in zooplankton growth compared to biochemical limitation (e.g., contents of unsaturated fatty acids) (DeMott et al. 1998; Boersma 2000). The very P-deficient algae (C : P = 900 in molar) were used as the P-limited diet to minimize the effects of biochemical limitation. *Daphnia* were fed with −P food (−P animals) and +P food (+P animals) for 5 d to achieve different C : P ratios in their bodies. The dietary ingestion, absorption, and efflux after incorporation were then examined in the +P and −P animals. During the efflux, losses of C and P through different physiological pathways (excretion, respiration, reproduction, and molting) were directly quantified. By comparison in *Daphnia* with contrasting nutrient status and feeding histories, the stoichiometric regulation was examined. Furthermore, we also investigated the recovery of animals from P limitation by shifting diets into +P.

Materials and methods

Algae and zooplankton—All the experiments were conducted with a monoclonal laboratory culture of *D. magna*, which was originally obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. In our laboratory, the animals have been cultured in glass-fiber (GF/C) filtered pond water at 23.5°C with a 14 : 10 light: dark cycle and fed *Chlamydomonas reinhardtii* at a saturating food concentration on a daily basis for over 6 yr. The total P, C, and C : P ratio in the pond water was 8.73 ± 0.45 μg L^{-1}, 321 ± 24 μg L^{-1}, and 92 ± 2 in molar, respectively. The total P in the filtered pond water was 0.48 ± 0.05 μg L^{-1}. The algae *C. reinhardtii* for *Daphnia* maintenance were grown in the artificial WC medium (Guillard 1975) for freshwater phytoplankton culture prepared from ultrapure water under a light intensity of 100 μmol photons m^{-2} s^{-1}. All experiments were conducted at 23°C in 0.22-μm-filtered and then autoclaved pond water in order to minimize the presence of bacteria that may potentially affect the recycling of dissolved C and P.

Cultures of P-deficient *C. reinhardtii* (C : P ratio of 930) (hereafter termed −P algae) were obtained from 5 d of semicontinuous culture. Algal cells at the exponential phase from the batch culture were suspended into the modified WC medium but with a P concentration of 1 μmol L^{-1}. Every 24 h, the cells were collected by centrifugation at 2,000 rpm (836 × g) for 20 min and resuspended into the new medium. At the end of the algal culture, the cells were harvested by centrifugation under the same condition. The P-sufficient algae (C : P ratio of 90) (hereafter termed +P algae) were grown in a batch culture with a standard WC medium and after 7 d were collected by centrifugation at 3,500 rpm (2,561 × g) at 15°C for 10 min. To radiolabel the −P algae, the cells were resuspended in the modified WC medium (400 mL) without P at 2.5×10^5 cells mL^{-1}, and then the carrier-free radioisotopes ^{33}P (as $H_3{}^{33}PO_4$) and ^{14}C (as $NaH^{14}CO_3$ in 10 mol L^{-1} NaOH) were spiked simultaneously. Radioactivity additions were 100–150 μCi L^{-1} (3.7–5.6 × 10^6 Bq L^{-1}) for ^{33}P and 80–100 μCi L^{-1} (3.0–3.7 × 10^6 Bq L^{-1}) for ^{14}C. After 72 h of radiolabeling, the cells were considered uniformly labeled, and the C : P ratio did not differ significantly from that before the radiolabeling (confirmed by direct measurements). Procedures for dual radiolabeling of the +P algae were the same as those for the −P ones, except that the standard WC medium was used. The radiolabeled algae (with specific radioactivity of 1.6–4.5 × 10^7 cpm mg^{-1} C and 1–3 × 10^7 cpm μg^{-1} P) were finally harvested by centrifugation and added to the filtered pond water before fed to *Daphnia*. At the same time, the C and P contents of the suspended algae were measured, and the final molar C : P ratio was calculated.

P-limited and P-sufficient Daphnia—A cohort of neonates from the same brood was cultured using the +P algae at a food concentration of 10^5 cells mL^{-1} (1.4 mg C L^{-1} or 40 μg P L^{-1}) to the adult stage, and neonates produced after the third brood within 24 h by these *Daphnia* were collected (about 600 neonates). They were fed +P algae from birth until they reached the age of 6 d. Half the animals were then fed −P algae at 10^5 cells mL^{-1} (1.5 mg C L^{-1} or 4.2 μg P L^{-1}) (hereafter termed −P *Daphnia*), and the other half was still on the +P algae (hereafter termed +P

Daphnia). After 5 d, 10 individuals from each culture were transferred and dried overnight at 60°C for both C and P measurements. The resulting C : P ratio in the cohort of +P *Daphnia* was ~72 as compared to ~126 in the −P *Daphnia* (see Results). The remaining animals were subsequently used in the following feeding experiments.

Ingestion rate and gut passage time of food particles—Ingestion rates of +P animals feeding on +P algae and −P animals feeding on +P and −P algae were measured in this experiment, which was performed with six replicates for each treatment. In each replicate, 10 individuals were incubated with corresponding food particles at food concentration of 10^5 cells mL^{-1} (1.4 mg C L^{-1} or 40 μg P L^{-1} for +P food and 1.5 mg C L^{-1} or 4.2 μg P L^{-1} for −P food) in 100 mL filtered pond water for 2 h. The food concentration was measured using a Coulter counter (Beckman) immediately before and at the end of incubation. The ingestion rate was computed as the decrease in the amount of food particles divided by incubation time.

The time required for *Daphnia* (11 d old) to produce their first feces was also examined with six replicates for each treatment. After evacuation of gut contents for 2 h in the absence of food particles, each free-swimming individual was placed in a well dish containing 5 mL of filtered pond water with corresponding food particles at 10^5 cells mL^{-1}. The feces production was observed continuously under the microscope. The time required for the first appearance of feces was defined as the gut passage time (GPT) of food particles.

Short-term feeding: Absorption efficiency and loss of C and P—Both algal quality and daphnid body stoichiometry (in terms of C : P ratio) obtained from different prefeeding conditions were considered in this experiment. The control treatment was the +P *Daphnia* fed +P algae. The P-limited treatment was the −P *Daphnia* fed −P algae, whereas the recovery treatment was the −P *Daphnia* fed +P algae. In each treatment (with three replicates), *Daphnia* with similar body sizes (11 d old) were first acclimated for 30 min under corresponding food conditions (nonradioactive) at a food concentration of 10^5 cells mL^{-1}. A total of 26 individuals from each replicate were then fed the corresponding radiolabeled algae at a food concentration of 10^5 cells mL^{-1} in 130 mL of filtered pond water for 5 min, which was shorter than GPT (about 10–15 min), to avoid the defecation of radioactive feces. After the pulse feeding, the animals were removed by a mesh and rinsed with 0.22-μm-filtered pond water. After placing them in nonradioactive water, four individuals were immediately picked from each replicate and transferred into vials containing 0.5 mL of 1 mol L^{-1} NaOH to solubilize the tissues (Xu and Wang 2003). The animals were later counted for their radioactivity (representing the total amount of radioactivity ingested by *Daphnia* during the 5-min feeding period). The remaining 22 individuals were placed in 110 mL of new pond water to purge their ingested radiolabeled food with nonradioactive food under the same food conditions corresponding to those used during the pulse feeding. The pond water was changed after 1, 2, 5, 8, and 12 h to avoid the possibility of ^{33}P and ^{14}C recycling. At each time point, four individuals were taken for measurements of radioactivity, a 3-mL aliquot was pipetted into plastic vials for measurements of total P (as $T^{33}P$) or total C (as $T^{14}C$), and another 3 mL were filtrated through a 0.22-μm Millipore membrane to remove the particulate materials before counting for dissolved P (as $D^{33}P$) or C (as $D^{14}C$). To measure the respired $^{14}CO_2$, a 13-mL aliquot was collected, and measurement of $^{14}CO_2$ followed the methods described by Wang and Guo (2001). Briefly, the sample was acidified at pH <2 and then bubbled with N_2. The released $^{14}CO_2$ was trapped in 0.5 mL of 2 mol L^{-1} NaOH. All procedures were conducted immediately to avoid the utilization of ^{33}P and ^{14}C by algae and bacteria.

Long-term feeding: Efflux and loss budgets of C and P—This experiment similarly included control (+P), recovery, and −P treatments, each with three replicates. After 1 d of the differential feeding (described above), *Daphnia* were fed with dual radiolabeled algae with corresponding C : P ratios (48 individuals). Thus, a total of 4 d of dual radiolabeling was performed (out of 5 d of differential feeding). They reached the age of 11 d when those daphnids were consequently collected for later experiments. After rinsing with filtered pond water, four individuals were removed for measurement of the initial radioactivity in the animals (before the depuration). The remaining 44 individuals were subsequently depurated in 220 mL of filtered pond water with the addition of nonradioactive food with the same C : P ratios as that used during the labeling period (for +P algae: 900 ± 40 vs. 930 ± 15 in radiolabeling, $p = 0.307$; for −P algae: 91 ± 4 vs. 90 ± 7 in radiolabeling, $p = 0.498$, *t*-test) for 3 d.

During the 3-d depuration period, the animals were fed with new food at a food concentration of 10^5 cells mL^{-1} for 2 h every 8 h instead of continuous feeding to avoid recycling of ^{33}P and ^{14}C released by *Daphnia* back to *C. reinhardtii*. The daphnids were then transferred to new filtered pond water to continue the depuration during the remaining 6 h. Each time when the food was added, four individuals were taken for measurement of radioactivity as described above. Molts, feces, and neonates or nonviable eggs produced by the adults were carefully collected with a wide-mouth pipette and then rinsed with filtered pond water to exclude contamination of ^{14}C and ^{33}P that were absorbed on the surface (which may bring overestimation of radioactivity of samples). The short interval of measurements (every 8 h) ensured that any molts collected were produced by the adults within the past 8 h. One 3-mL water subsample was removed for measurements of $D^{33}P$ and $D^{14}C$, while another 3 mL of water were measured for $T^{33}P$ and $T^{14}C$. Another 13 mL of water were also taken to quantify the respired ^{14}C.

The efflux rates of ^{33}P and ^{14}C and the growth rates of the animals were needed in calculating the specific radioactivity of the animals. To measure the somatic growth rates of adults and neonates, about 100 adults or 150 juveniles from each treatment were fed under the same conditions as those in the efflux experiments (but nonradioactive). Ten adults or 15 juveniles were collected at 0, 24, 48, and 72 h to measure the P and C contents in the

animals. The somatic growth rates were then calculated as the slope of regression between the natural log of P or C against the time of growth.

Chemical and data analysis—Algae that were resuspended in the feeding beakers were collected by filtration onto precombusted (500°C, 4 h) GF/F filters and dried at 80°C in an oven for 2 d. *Daphnia* were also dried at 80°C for 2 d. The carbon contents of algae and *Daphnia* were measured with a CNH analyzer (Series II CHNS/O Analyzer 2400, PerkinElmer Instruments). The P contents were measured by molybdate blue reduction (Murphy and Riley 1962) after a hot acidic oxidative hydrolysis with 5% $K_2S_2O_8$, and the tissues were dissolved with H_2O_2 at 130°C.

The radioactive animals were solublized in 0.5 mL 1 mol L^{-1} NaOH at 60°C overnight. The cocktail (PerkinElmer) was then added, and the radioactivity was measured by a Wallac 1414 liquid scintillation counter (Wallac Oy) using the external standard ratio method. Dual counting of ^{14}C and ^{33}P followed the method described by Lehman (1993).

The absorption efficiency was defined as the fraction of digestive products taken up across the gut wall (Penry 1998). Since feces egestion accounted for the majority of total body ^{14}C and ^{33}P loss within the first 2 h of depuration (because of the high food concentration used in the feeding experiment), the absorption efficiency (AE) was calculated as the *y*-intercept of the linear regression between the natural log of the percent of C and P retained in the animals and the time of depuration (2–12 h) (Wang and Fisher 1998). The exact time at which assimilation was complete was difficult to be determined from this study.

Data obtained in the short-term experiments can be conveniently used to estimate the growth efficiency. The growth can be calculated as the ingestion – release (Olsen et al. 1986; DeMott et al. 1998), where the release includes defecation, excretion (C and P), and respiration (for C). The gross growth efficiency (GGE) was calculated as growth divided by the ingestion, and the net growth efficiency (NGE) was calculated as growth divided by absorption. The percent of C or P released into each compartment was calculated as the radioactivity detected in each compartment divided by that in the total carbon or phosphorus (sum of all compartments, including dissolved phosphorus or dissolved organic carbon [DOC], molts, and neonates). The average mass specific radioactivity of animals during a certain interval (8 h) in the efflux experiments was calculated by

$$\overline{S} = \left(\int_{t_1}^{t_2} S_0 \cdot e^{-(k+g)\cdot\Delta t} \right) \Big/ \Delta t \qquad (1)$$

where t_x is the time of sampling, Δt is the interval between t_2 and t_1, S_0 is the initial specific activity, k is the efflux constant rate, and g is the specific growth rate of the animals.

One-way analysis of variance (ANOVA) was performed to compare different parameters (absorption efficiency, ingestion rate, GPT, efflux constant rate, relative distribution of C or P loss, and mass-specific loss rate) among control, recovery, and P-limitation treatments, followed by a Tukey post hoc test. To meet the assumption of normality for an ANOVA, the percentage data were arcsin transformed, and natural log transformations were applied to other data where appropriate. Student's *t*-test was used to test the difference in C and C : P ratios between +P and −P animals and algae. The level of significance for all tests was $\alpha = 0.05$.

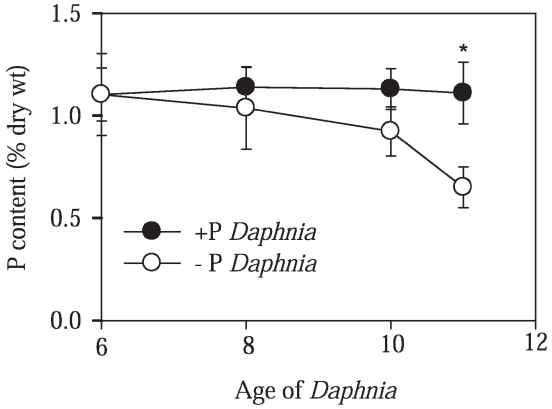

Fig. 1. The P contents of *Daphnia magna* under different dietary phosphorus conditions. +P: phosphorus sufficiency; −P: phosphorus deficiency. * indicates significant difference between the two treatments (*t*-test, $p < 0.05$). Data are means ± SD ($n = 2$).

Results

After 5 d of differential feeding, the P content was 1.11 ± 0.15% of dry weight for the +P animals and 0.65 ± 0.10% for the −P animals ($p < 0.05$, *t*-test) (Fig. 1). The body C : P ratio differed by about twofold between these two treatments (72 ± 9 for the +P animals and 126 ± 12 for the −P animals, $p < 0.05$, *t*-test). For both short- and long-term experiments, the sum of the radioactivity in each compartment and the loss of P and C from the animals were compared. The mass balance of the lost P and C was generally in the range of 95–115%, suggesting that P or C loss from the animals could be traced through measurements of each compartmental loss (e.g., excretion, molting, reproduction, and respiration).

Ingestion rate and gut passage time of food particles—Ingestion rate varied in *Daphnia* following the sequence: control > recovery treatment > deficiency treatment in terms of particle collection and C or P ingested ($F_{2,6} = 168.06$, $p < 0.001$, for particle; $F_{2,6} = 143.70$, $p < 0.001$, for C; $F_{2,6} = 52{,}050$, $p < 0.001$, for P; one-way ANOVA and Tukey post hoc test) (Table 1). The +P animals had significantly shorter GPT than the −P animals, which had comparable GPT with different diets (Table 1).

Absorption and growth efficiency of C and P after pulse feeding—Over the 12 h of depuration, the loss of ingested C from the daphnid body was initially fast and then slowed down (Fig. 2). The +P animals had the lowest C (15%) and P (36%) AEs, whereas the −P animals had the highest C and P AEs on −P food ($p < 0.001$, $F_{2,6} = 410.9$,

Table 1. The ingestion rates (IR), gut passage time of food particles (GPT), absorption efficiencies (AE) of carbon and phosphorus, and their ratios in the +P and −P *Daphnia magna* feeding +P or −P *Chlamydomonas reinhardtii*. Data are means ± SD (n = 6 for IR and GPT; n = 3 for AE).

Treatment		Ingestion rate			GPT	AE (%)		C : P	
D. magna	*C. reinhardtii*	Particle ($\times 10^6$ cell d^{-1})	C (μg d^{-1})	P (μg d^{-1})	(min)	C	P	Algae	Absorbed
+P	+P	1.52±0.08	20.5±1.0	0.61±0.002	6.9±2.3	15.1±1.3	36.1±1.2	90	38±5
−P	+P	1.04±0.05	14.0±0.7	0.42±0.0011	14.5±3.4	31.3±0.9	35.6±3.0	90	79±8
−P	−P	0.67±0.03	8.70±0.4	0.024±0.0001	13.3±3.1	46.3±1.6	52.5±5.0	930	820±80

for C AE; p = 0.001, $F_{2,6}$ = 23.4, for P AE; one-way ANOVA and Tukey post hoc test) (Table 1). When the diets shifted from −P to +P, the AEs of C and P in −P daphnids decreased significantly. The ratio of absorbed C and P was calculated by combining the C and P AEs with the C : P ratios of food (Table 1), showing significant differences among treatments ($p < 0.001$, $F_{2,6}$ = 268.7, one-way ANOVA). The −P animals absorbed materials at C : P ratios close to their −P or +P diets, whereas the +P animals absorbed carbon and phosphorus at C : P ratios significantly lower than that in the +P diets.

Daphnia in the P-limited treatment had the highest GGE and NGE in terms of C and P (p = 0.001, $F_{2,6}$ = 31.3, for GGE of C; p = 0.002, $F_{2,6}$ = 20.1, for NGE of C; p = 0.003, $F_{2,6}$ = 18.2, for GGE of P; p = 0.001, $F_{2,6}$ = 34.5, for NGE of P; one-way ANOVA and Turkey post hoc test) (Fig. 3). The recovery animals had GGEs of C and P similar to the control animals but had the lowest NGEs of C and P (Tukey post hoc test) (Fig. 3).

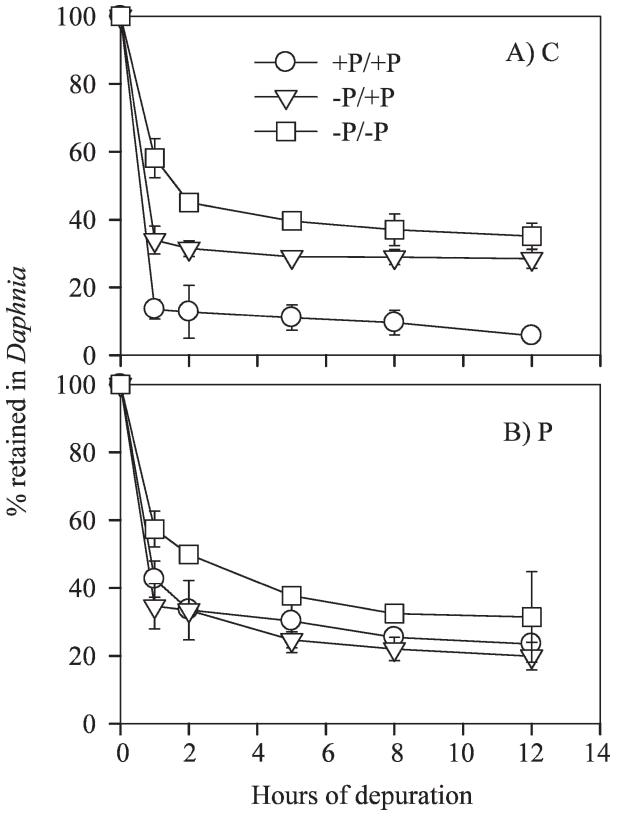

Fig. 2. Retention of (A) ingested carbon and (B) phosphorus in *Daphnia magna* with different body C : P ratios following a pulse feeding on algae *Chlamydomonas reinhardtii*. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. Data are means ± SD (n = 3).

Efflux and loss budget of C and P—During the 3 d of depuration, the body C was lost from *Daphnia* gradually, and at the end of depuration, 30–60% of ^{14}C remained in the animals (Fig. 4). The relationship between ^{14}C remained in *Daphnia*, and the depuration time was well fitted by a one-compartment model; thus, the efflux rate constant was calculated from the linear regression between the natural log of ^{14}C retained in the animals and the time of depuration (1 d onward; $p < 0.0001$ for all the treatments) (Table 2). The pattern of ^{33}P loss was similar to that of ^{14}C, and the +P animals had 50% of P retention, while the −P animals fed −P food had a much higher P retention (79%). Similarly, the efflux rate constant of P was calculated ($p < 0.0001$ for the treatments) (Table 2). The calculated C efflux rate constant was the highest for −P animals fed −P diet (0.41 ± 0.04 d^{-1}), but the P efflux constant was the lowest (0.10 ± 0.02 d^{-1}) among the three treatments (p = 0.005, $F_{2,6}$ = 14.8, for C; $p < 0.001$, $F_{2,6}$ = 73.5, for P; one-way ANOVA). The relative efflux rate of C

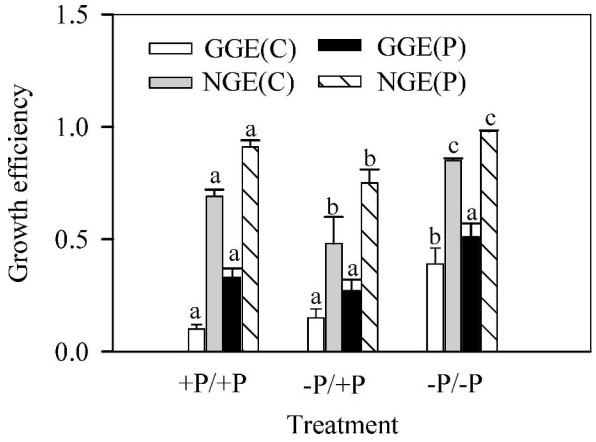

Fig. 3. Gross growth efficiency (GGE) and net growth efficiency (NGE) in terms of carbon and phosphorus in *Daphnia magna* with different body C : P ratios. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. Data are means ± SD (n = 3). Different letters above the bars represent significant difference between the two treatments for each growth efficiency.

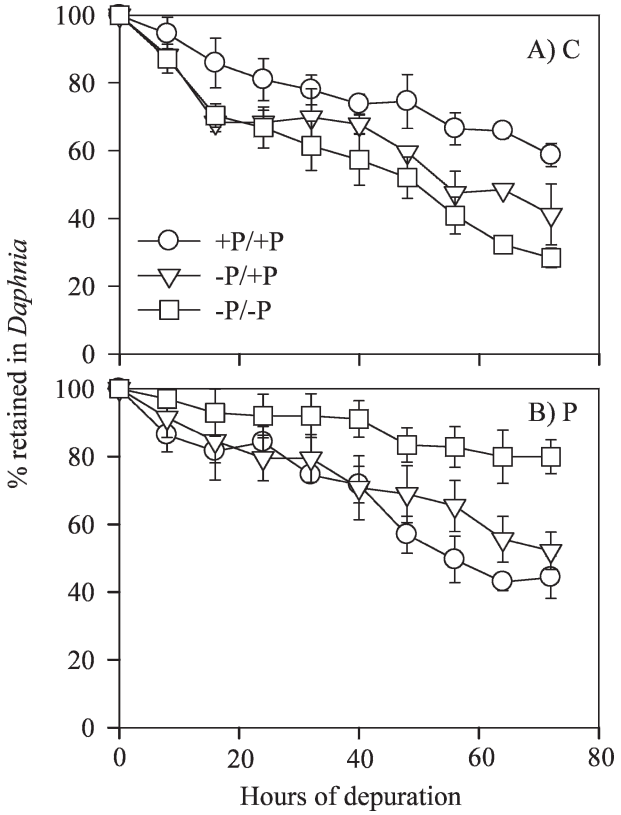

Fig. 4. Retention of (A) structural carbon and (B) phosphorus in *Daphnia magna* with different body C : P ratios during 72-h depuration period. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. Data are means ± SD (n = 3).

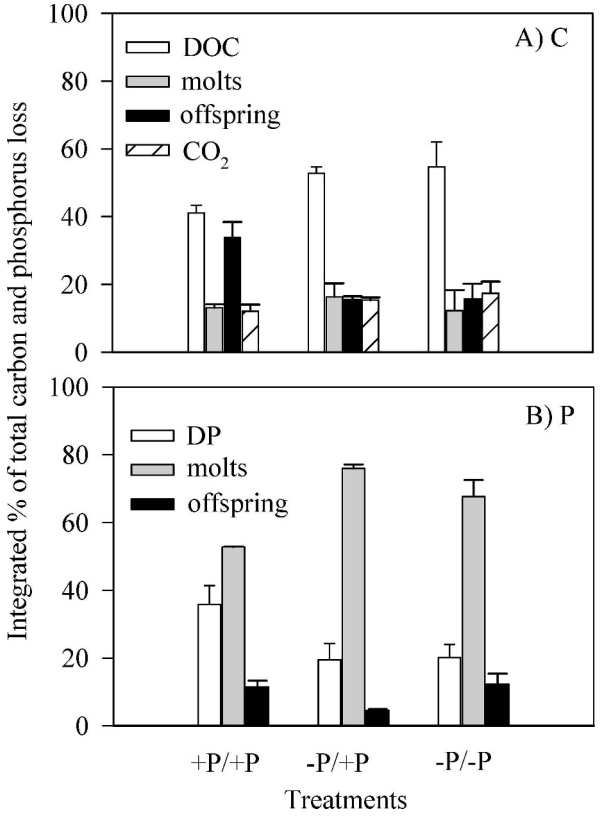

Fig. 5. Relative contribution of each route (DOC: dissolved organic carbon; CO_2: respiration; DP: dissolved phosphorus) to (A) carbon or (B) phosphorus loss from *Daphnia magna* with different body C : P ratios over 72-h depuration period. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. Data are means ± SD (n = 3).

to P was also calculated by dividing the P efflux rate constant with the C efflux rate constant (Table 2), and all treatments differed significantly from each other ($p < 0.001$, $F_{2,6} = 8.8$). For the +P animals, the body C loss was slower than the P loss (0.42 ± 0.10). However, the relative C-to-P efflux in the −P animals fed −P food was the highest (4.1 ± 0.4), suggesting that C was lost much faster from the body than P in this treatment.

Loss of body C was further partitioned into four compartments including CO_2 (respiration), DOC, offspring (reproduction), and molt. The averaged contribution of each compartment to the overall C loss was calculated by integrating the entire depuration period (Fig. 5). For the +P animals, DOC and reproduction were the two most important compartments with comparable contributions to the total loss; together they accounted for nearly 70% of C released ($p < 0.001$, $F_{3,8} = 84.2$; one-way ANOVA). For the −P animals, the DOC was the predominant compartment (45–52%). There was no significant difference in the contributions of other compartments such as CO_2 and molts (including reproduction in the −P animals) among different treatments (Table 3; Tukey post hoc test). Absolute C loss from *Daphnia* was calculated by dividing the integrated radioactivity of each compartment with the specific radioactivity of the animals (Eq. 1), and the mass-specific loss rate was calculated by dividing the absolute loss with the average dry weight and depuration time (Fig. 6). Overall, the ranges of the mean C loss rate in the compartments of DOC, molts, reproduction, and CO_2 were 1.01–1.85, 0.32–0.63, 0.45–0.83, and 0.30–0.58 μg C

Table 2. Efflux rate constants of carbon and phosphorus and their relative ratios in the +P and −P *Daphnia magna* feeding on +P or −P *Chlamydomonas reinhardtii*. Data are means ± SD (n = 3).

Treatment		Efflux rate constant (d^{-1})		
D. magna	*C. reinhardtii*	C	P	Relative C to P efflux
+P	+P	0.16±0.09	0.38±0.04	0.42±0.1
−P	+P	0.34±0.02	0.24±0.02	1.4±0.03
−P	−P	0.41±0.04	0.10±0.02	4.1±0.4

Table 3. One-way analysis of variance applied to the relative distribution of P and C loss and the mass-specific loss rates through different pathways during the efflux period in *Daphnia magna* (df: degree of freedom; *p*: probability). DOC: dissolved organic carbon; CO_2: respiration; DP: dissolved phosphorus; Ctrl: +P *Daphnia* fed +P algae; −P *Daphnia*: P-deficient *Daphnia* fed −P algae; recovery: −P *Daphnia* fed +P algae. Statistical significances in Tukey post hoc test are indicated by * $p<0.05$; ** $p<0.01$; *** $p<0.001$. In most cases, the recovery treatment did not differ significantly from the P deficient treatment, but was significantly different from the control treatment.

			Relative distribution			Specific loss rate		
	Compartments	df	$F_{2,6}$	*p*	Tukey post hoc test	$F_{2,6}$	*p*	Tukey post hoc test
C loss	DOC	2	7.9	0.021	Ctrl < −P *Daphnia**	25.9	0.001	Ctrl < −P *Daphnia**
	Molts	2	0.8	0.504		8.3	0.019	Ctrl < −P *Daphnia**
	Offspring	2	23.4	0.001	Ctrl > −P *Daphnia***	4.3	0.069	
	CO_2	2	4.1	0.077		22.5	0.002	Ctrl < −P *Daphnia**
P loss	DP	2	9.3	0.014	Ctrl > −P *Daphnia**	21.6	0.002	Ctrl > −P *Daphnia***
	Molts	2	43.8	<0.001	Ctrl < −P *Daphnia***	27.0	0.001	Ctrl > −P *Daphnia***
	Offspring	2	10.7	0.011	Ctrl = −P *Daphnia* > recovery*	192.2	<0.001	Ctrl > −P *Daphnia****

mg^{-1} h^{-1}, respectively. The −P animals had a higher DOC release rate and respiration rate than the +P animals, and the latter lost more C through reproduction (Table 3).

The averaged contribution of each compartment to the P loss from animals was calculated by integrating the entire depuration period (Fig. 5). The molts represented the predominant route for all three treatments, accounting for 53–76% of the total P loss, followed by DP with a contribution of 20–36% of the total P loss ($p < 0.001$, $F_{2,6} = 50.7$, 572.7, 145.8 for control, recovery, deficiency treatment; one-way ANOVA). More P was lost by molts for −P *Daphnia*, while the DP had a lower contribution (Fig. 5). The relative ratio of molts over DP was smaller in the +P animals compared with that in the −P animals. The mass-specific loss rate was calculated from the specific radioactivity of P in the animals, dry weight, and depuration time (Fig. 6). In the control animals, the loss rates through DP release, reproduction, and molting were 0.053, 0.017, and 0.099 μg P mg dry wt^{-1} h^{-1}, while those in the −P animals were 0.0066–0.011, 0.0028–0.0035, and 0.026–0.034 μg P mg dry wt^{-1} h^{-1}, respectively. Overall, the specific P loss rates through all compartments were higher in the +P animals than those in the −P animals, in which the difference in diets (+P and −P) made no difference (Table 3).

The C : P ratios (molar) of total dissolved releases, reproduction, and molts were calculated throughout the depuration period (Fig. 7). Within a treatment, molts had the lowest C : P ratio, and the C : P ratios in molts differed significantly between treatments (8.0 ± 0.3 vs. 36 ± 7 and 61 ± 20 in control, recovery, and deficient treatments, $p = 0.005$, $F_{2,6} = 14.8$; one-way ANOVA and Tukey post hoc test). The C : P ratios of offspring were comparable between

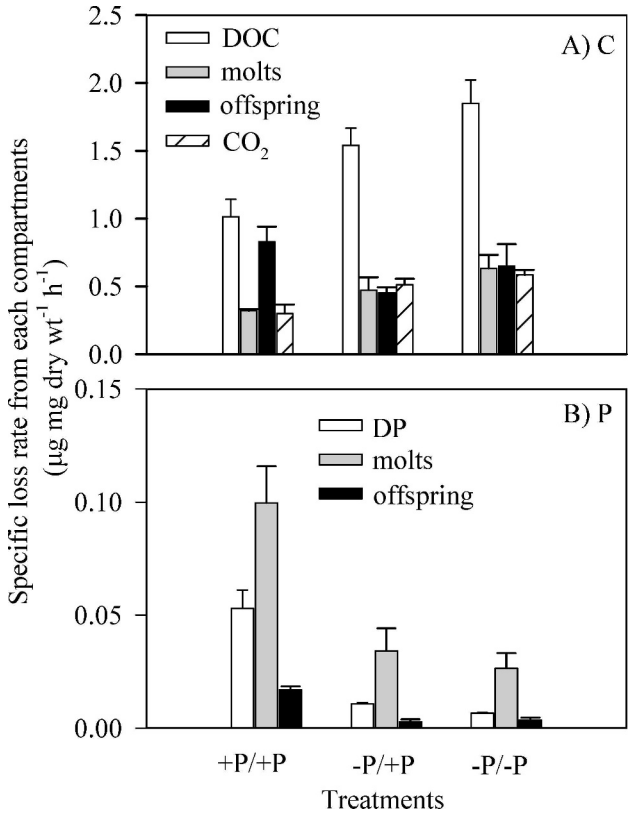

Fig. 6. Mass specific rate of (A) carbon and (B) phosphorus loss from *Daphnia magna* with different body C : P ratios during 72-h depuration period. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. DOC: dissolved organic carbon; CO_2: respiration; DP: dissolved phosphorus. Data are means ± SD ($n = 3$).

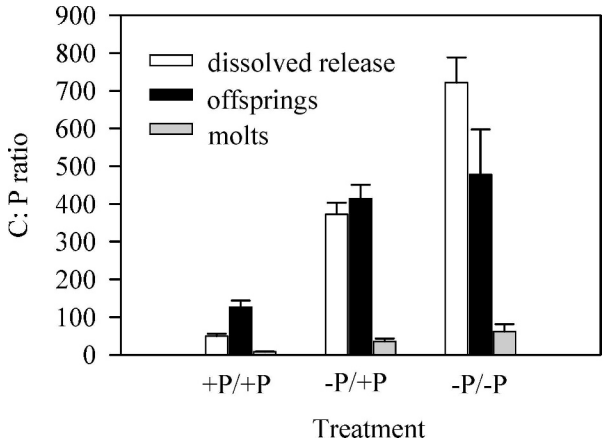

Fig. 7. C : P ratios of different compartments of loss of carbon and phosphorus from *Daphnia magna* with different body C : P ratios. +P/+P: +P *Daphnia* feeding +P algae; −P/+P: −P *Daphnia* feeding +P algae; −P/−P: −P *Daphnia* feeding −P algae. Data are means ± SD ($n = 3$).

the −P *Daphnia* with +P and −P diets (414 ± 36 vs. 477 ± 120), and both were higher than those in +P *Daphnia* (126 ± 17; $p = 0.002$, $F_{2,6} = 19.6$; one-way ANOVA). Dissolved release by the control animals had remarkably lower C : P ratios than those from −P animals, which had the highest C : P ratios when fed with the −P diets (49 ± 6 vs. 372 ± 30 and 721 ± 67 in control, recovery, and deficient treatments, $p < 0.001$, $F_{2,6} = 184.8$; one-way ANOVA and Tukey post hoc test).

Discussion

Ingestion and absorption of C and P—Feeding on diets of different qualities for 5 d resulted in a significant difference in P content in *Daphnia* (1.11% under +P vs. 0.65% under −P). Similarly, DeMott (2003) found that there was 2× difference in the P content in *Daphnia* under contrasting feeding conditions. In another study, DeMott et al. (1998) showed that the P content of *Daphnia* (4 d old initially) dropped from 1.53% to 1.11% after 5 d of feeding on −P diets. In our study, the decline of the specific P content of *Daphnia* was small until the fourth day of feeding, whereas DeMott et al. (1998) found that the P content dropped abruptly within the first day of feeding. Such discrepancy may have been due to the differences of daphnid ages when the feeding started (e.g., 6 d old in our study vs. <24 h and 4 d old in DeMott et al. 1998) since the younger animals may be more sensitive to P limitation (Sterner and Hessen 1994).

The decrease in ingestion rate under P limitation in the present study was consistent with the findings in previous studies (van Donk and Hessen 1993; DeMott et al. 1998; He and Wang 2007). Van Donk and Hessen (1993) attributed such decrease to the resistance of nutrient-limited algae against zooplankton grazing. However, the comparable GTPs in −P animals on +P and −P diets do not support this explanation. The long history of feeding by the −P animals may have accounted for the decrease in ingestion, as feeding on −P algae may be an energy-consuming process.

The AEs of C and P (15–46% for C and 36–53% for P) were lower than those obtained by DeMott et al. (1998) (>60% for P and >50% for C) because a higher food concentration was applied in the present study (1.4–1.5 mg C L^{-1} vs. 0.5–1 mg C L^{-1} by DeMott et al. 1998). Higher AEs of C and P were found in animals under −P-limited conditions, and comparable P absorption was found between the −P and +P animals fed +P algae. Such increase in AE may partly be due to the low ingestion rate of the −P animals. The relationship between P AE, preconditioning of animals, and food quality in the present study is consistent with the findings on the incorporation efficiency of P in *D. magna* by Boersma and Wiltshire (2006), who used different incubation regimes (6 d of prefeeding + 3 h of incubation with radiolabeled food particles). In their study, the incorporation rate of −P algae by −P animals was 80% higher than that of +P algae, and the latter were incorporated by both −P and +P animals with comparable efficiencies. However, DeMott et al. (1998) found that long acclimation time (36–40 h) resulted in a decrease of both C and P AEs. Although there was no direct information about the body stoichiometry of *Daphnia* in that study, the body C : P ratio at least increased after 36 h of feeding on P-deficient food (DeMott et al. 1998; DeMott 2003).

The growth efficiency of P was consistently higher than that of C, which is in agreement with DeMott et al. (1998). The −P animals showed higher growth efficiency for both C and P than the +P animals, probably because of the decrease in ingestion rate under the −P condition, which induced a higher utilization efficiency to accumulate more P with an attempt to increase the body P to an optimal level. However, the consistent trend of growth efficiency of C and P was not found by DeMott et al. (1998). In their study, the gross growth efficiency but not the net efficiency of C decreased in −P conditions, whereas both efficiencies of P showed a unimodal response with increasing P limitation and were the highest at intermediate values. The reason for the discrepancy is not known, although it is noted that their study used a much shorter acclimation period than that used in our study (2 d in the study by DeMott et al. vs. 5 d in our study).

Integrating the ingestion, digestion, and absorption processes in *Daphnia* under contrasting phosphorus conditions, our results indicated that P deficiency promoted the successive changes during the preabsorption and absorption processes, such as the prolonged gut passage time of food particles and an increase in absorption efficiency and growth efficiency. However, it was questionable whether the stoichiometric regulations at these two levels were sufficiently effective, because the −P animals absorbed C and P at the C : P ratios much higher than their body C : P ratios.

Efflux of carbon and phosphorus—The efflux rate constant of C (0.16 d^{-1}) in +P *Daphnia* was comparable to our earlier measurements on the juveniles (0.12–0.16 d^{-1}; He and Wang 2006). The −P animals had a higher C but lower P efflux than the +P animals. Conversely, the C efflux slowed down while the P efflux picked up during the course of recovery of −P *Daphnia* from P limitation. C and P were lost through different pathways, including excretion, reproduction, molting, and respiration in this study. The changes of mass-specific loss rate and C : P ratio in each pathway may provide clues for the stoichiometric regulation of the animals in response to P deficiency.

The C loss by respiration in +P *Daphnia* accounted for 2% of total C loss or 9% of absorbed C, which was within the range found for different species of zooplankton (5–30% of absorbed C was lost by respiration; Sorokin 1968; Hargrave 1970; Conover and Francis 1973). Under P deficiency, the percentage of carbon loss increased to 8% of total C loss or 18% of absorbed C. In terms of mass-specific respiration rate, the average respiration rate increased in the −P animals compared with the +P animals. The respiration loss increased as body C increased (1.6% vs. 2.7% or 3.1% of body C d^{-1}). Darchambeau et al. (2003) reported an increase in respiration rate (from 25% to 39% of body C) when the +P daphnids were fed the −P

food. The much higher percentage of body C consumed through respiration in their study may partially be explained by that fact that they used juveniles (which have a higher ingestion rate and body-specific respiration rate than adults).

DOC was produced by excretion during the long-term depuration period, while DP release included both the excretion and the molting fluids (Peters and Rigler 1973), which was verified by the finding of peaks in the P release from molting *Daphnia* (Scavia and McFarland 1982). The DOC release rate also increased in the −P animals, from 5.4% to 8.2–9.9% of body C. Consistently, Darchambeau et al. (2003) found that the excretion rate increased from 5.7% to 13.4% of body C d^{-1} in the +P animals when they were shifted from +P to −P diets. The DP release rate in the +P animals (53 ng mg^{-1} h^{-1}) was close to the low ends of the broad range of P release rates reported in the literature (20–1,100 ng P mg^{-1} h^{-1}; Rigler 1961; Peters 1975; Wen et al. 1994). Contrary to DOC, the DP excretion rate decreased significantly under −P deficiency by 80–88%. When expressed by the body P, the DP decreased from 11% to 2–4% of body P d^{-1}. Decrease in DP release had also been reported in the study by Frost et al. (2004), but these authors found that the DOC release was independent on food quality.

The C : P ratio in the dissolved excreta of +P *Daphnia* (49 ± 6) was similar to C : P ratios in *Daphnia* body (64 ± 8) but increased greatly for the −P *Daphnia* (372 and 721 with +P and −P diet), highlighting that the C : P ratio of soluble release may be dependent on both the dietary C : P ratios and the body C : P ratios (reflecting the feeding history). The stoichiometric theory predicted that nutrient recycling in zooplankton should be tightly coupled with the resource nutrient ratios (Sterner and Hessen 1994; Elser et al. 1996). More recently, both modeling and experimental measurements showed the dependence of DOC : P release ratio on food C : P ratio (Frost et al. 2004). However, these previous studies did not directly consider the feeding history of P-deficient animals on the release stoichiometry, which may influence body elemental ratios and composition of grazers.

For +P *Daphnia*, C was lost through molting at a rate of 0.32 μg mg dry wt^{-1} d^{-1} (or 1.7% of body C d^{-1} assuming that C content was 45% of dry wt; Hessen 1992), comparable to the estimation (2.5% of body C d^{-1}) by Hessen and Rukke (2000). If the body C content remained consistent even under P deficiency, the C loss increased to 2.5–3.4% of body C d^{-1}. In terms of P loss through molting, it represented the predominant loss pathway for both +P and −P animals. Specific P loss rate through molting (0.1 μg mg dry wt^{-1} h^{-1}) was nearly identical to our previous measurements (88–98 ng mg dry wt^{-1} h^{-1}; He and Wang 2007). A simple conversion of P loss rate showed that P loss rate was 21.7% of body P d^{-1} in +P *Daphnia*, which was much higher than the previous estimation (~7.5% body P d^{-1}) by Hessen and Rukke (2000). The loss rate decreased to 9.8–12.6% of body P d^{-1} in –P animals fed +P or −P diets. Consequently, the C : P ratio of molts increased dramatically from 8 (in molar) under P sufficiency to 36–61 under P deficiency. This C : P ratio for the +P animals was notably lower than 65 estimated by Hessen and Rukke (2000). To our knowledge, our study is the first to directly measure the C : P ratio of molts.

Reproduction in +P *Daphnia* represented a loss of 4.4% of body C and 3.7% of body P per day, and the loss decreased to 2.4–3.5% of body C and 1.0–1.3% of body P in the −P animals. Vrede et al. (1999) found that 20% of body P was allocated to offspring, and Ventura and Catalan (2005) found that as high as >30% of body C and 35% of body P were lost seasonally in *Daphnia pulicara* through reproduction. One possible explanation for our lower values could be that the animals were not fed continuously (even though they were fed at a high food concentration). Indeed, about 66% of the total offspring was in the form of nonviable eggs in +P animals, while the −P animals produced only nonviable eggs (100%). Recently, He and Wang (2007) reported a higher percentage of body P allocation in reproduction in the +P animals (12% of body P per day) under similar feeding regimes (noncontinuous feeding). One possibility for such discrepancy was the use of 14-d daphnids in our previous study, as compared to the 10-d daphnids used in our present study. Under any nutrient condition, the brood size increased with body length or age (Urabe and Sterner 2001). Furthermore, Urabe and Sterner (2001) also showed that the younger egg-bearing adults may be more vulnerable to the inferior conditions by producing more nonviable eggs. It was possible that the release of nonviable eggs was caused by the intermittent feeding regimes used in our study, which was done to avoid P recycling in our experimental systems. Interestingly, the investment of +P animals fed the −P food in reproduction found by He and Wang (2007) was very close to that of −P animals found in the present study.

The nonviable eggs resulted in a high C : P ratio of reproduction (126 in molar), which was comparable to the value (C : P of 120) reported by Færøvig and Hessen (2003). For the −P animals, the production of nonviable eggs was consistent with the speculation by Urabe and Sterner (2001) that producing nonviable eggs could be a pathway to deal with excessive C since the C : P ratio was remarkably higher in their nonviable eggs from −P animals (414 and 477 with +P and −P diet, respectively) than in those of +P animals (126) in this study.

The overall C : P ratios of different loss compartments of *Daphnia* in this study increased dramatically in the −P animals compared to +P animals (8- or 15-fold in DP, 3–4-fold in reproduction, and 4–7-fold in molts). In contrast, the body C : P ratio only doubled, suggesting that the body stoichiometric maintenance was achieved through all pathways by increasing C loss and decreasing P loss. It appeared that dissolved material release played the most important role in the change of the C : P ratio, consistent with the assertion by Anderson et al. (2005). Among all the possible pathways, respiration played a relatively minor role in body stoichiometric regulation.

The recovered −P animals consistently had intermediate values of AE, efflux, and mass-specific loss rate through all pathways, indicating that the change of body stoichiometry from +P to −P is a reversible process, which again supported the overall stoichiometric regulation. A similar

recovery experiment has been performed by DeMott (2003). In his study, when *Daphnia* were exposed to a P-deficient diet for 2 d and were then switched to P-sufficient food for another 2 d, the body C : P decreased rapidly. In our study, the decrease of body C : P ratio in animals was not as rapid as that found by DeMott (2003). One possible explanation for this difference could be the use of young adults (10–11 d old) in our study, as compared to neonates (<24 h) used in his study. An ontogenic difference in the P balance in *Daphnia* has been observed in our previous study (He and Wang 2007). Juveniles had higher AE and lower efflux rate than adults had. Furthermore, when the diets shifted to −P, the excretion rate decreased to a greater extent in juveniles than in adults (93% vs. 74%), also suggesting that juveniles required more P for their growth and thus were more sensitive to the change in P condition (Sterner and Hessen 1994).

In conclusion, *Daphnia* varied their body stoichiometry in response to different feeding histories (P sufficient and P deficient), and they showed different characteristics in C and P balances under different dietary P conditions. Under P limitation, the −P *Daphnia* increased the P AE but decreased the P efflux to retain the body P. On the other hand, the C loss rate increased in order to reduce the body C : P ratio. Furthermore, all the pathways for C and P loss (respiration, dissolved release, reproduction, and molting) were involved in stoichiometric regulation. Consequently, any decrease in the body P content of *Daphni*a as a result of P limitation may influence the relevant processes in freshwater environments. Viable offspring were not obtained from this study. The influence of maternal nutrient status on the body stoichiometry of the next generation is therefore unknown. Previous studies have shown a linear relationship between the body P content of mothers and daughters (DeMott et al. 1998; Boersma and Kreutzer 2002). On the other hand, the dynamics or robustness of successive generations would probably be affected by the body stoichiometry of the mothers, which could reflect the nutrient status of the mothers.

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