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The regulation of calcium in *Daphnia magna* reared in different calcium environments

Qiao-Guo Tan and Wen-Xiong Wang*

Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Abstract

The specific content, dissolved uptake rate, dietary assimilation efficiency (AE), and efflux rate constant (k_e) of calcium (Ca) were quantified in juvenile (4-d) and adult (10-d) *Daphnia magna* cultured in low (0.5 mg L^{-1})– and high (50 mg L^{-1})–Ca environments using a radiotracer technique. Daphnids raised in the high-Ca environment had higher Ca contents than did those raised in the low-Ca environment, and juvenile daphnids had higher Ca contents than adults. Uptake from solution was the dominant source (97–100%) of Ca for daphnids. The maximum influx rate (J_{max}) was higher in juvenile daphnids (3.24–4.10 mg g dry weight [wt]$^{-1}$ h^{-1}) than in adults (1.51–1.62 mg g dry wt^{-1} h^{-1}), while the influx rates were comparable in different Ca environments. The half-saturation concentration (K_m) was 2.51–5.58 mg L^{-1}. The AEs of Ca declined exponentially with increases in food concentrations, and lower AE was observed in the higher Ca environment. The k_e of Ca (0.83–1.98 d^{-1}) was the highest among the elements whose k_e had been quantified in *D. magna*, and it was 1.8–2.4 times higher in the high-Ca environment. Excretion into water was the dominant route (60–85%) of Ca release from daphnids; another 15–40% of Ca was lost as molts. The regulation of Ca in daphnids is mainly accomplished through adjusting their efflux but not their influx of Ca; their regulation ability is very limited, which may lead to a poor performance in daphnids in Ca-deficient water.

Cladocerans are ecologically important zooplankters in freshwater lakes. About 400 species have been identified, among which the *Daphnia* species have higher calcium (Ca) content (2–8% dry weight [wt]) than the nondaphnid cladocerans (0.2–0.4% dry wt) (Wærvågen et al. 2002; Jeziorski and Yan 2006). *Daphnia magna* is one of the *Daphnia* species with the highest Ca content (Wærvågen et al. 2002). The Ca content of *Daphnia* increases with the elevation of Ca concentrations in the water (Alstad et al. 1999; Rukke 2002; Jeziorski and Yan 2006), and it is independent of seasonal changes (Jeziorski and Yan 2006). In different *Daphnia* species, a positive relationship between Ca content and body size was observed (Wærvågen et al. 2002); however, within the same species, specific Ca content decreased with increases in body size (Alstad et al. 1999).

Calcium is mainly distributed in the exoskeleton of *Daphnia* in the form of carbonate and phosphate (Hessen and Rukke 2000). It is periodically lost with the discarded exuvia during molting. The average molting interval of *D. magna* is greatly affected by temperature. At 18°C, the molting frequency of juvenile *D. magna* is approximately every 2 d. This frequency decreases to every 4–5 d for adult daphnids (Hessen et al. 2000). Porcella et al. (1969) observed that the molting interval is more than 10 d at 10°C and that the interval decreases to 80 h and 60 h when the temperature increases to 20°C and 25°C, respectively. During one molting event, approximately 90% of the total body Ca is lost, including 50% into the water and 40% in the shed molt (Alstad et al. 1999), which indicates that *Daphnia* has no efficient mechanism for Ca storage. Therefore, in certain soft-water environments, *Daphnia* may confront Ca limitations and perform more poorly than its competitors who have lower Ca demands. It is not surprising that the abundance of *Daphnia* is positively correlated with Ca concentrations in the water (Hessen et al. 1995*a*). The Ca threshold for survival was found to be between 0.1 and 0.5 mg Ca L^{-1} (Hessen et al. 2000) for *D. magna* and between 0 and 2 mg Ca L^{-1} for *Daphnia galeata* (Rukke 2002). In *D. magna*, calcification is greatly affected when Ca is lower than 5 mg L^{-1} (Alstad et al. 1999). When Ca is lower than 10 mg L^{-1}, the egg production by *D. magna* is greatly reduced (Hessen et al. 2000). Juvenile *Daphnia* are more susceptible to Ca limitation than are adults (Hessen et al. 2000; Rukke 2002), although the underlying mechanism for this susceptibility is not clear.

The calcification process of *D. magna* is completed shortly after molting (i.e., within 24–48 h; Alstad et al. 1999). It seems unrealistic for daphnids to incorporate enough Ca via food ingestion to meet the demand for Ca for calcification. It stands to reason that the uptake of Ca from the water must lead to rapid calcification. Indeed, Cowgill et al. (1986) showed that the Ca content of daphnids is dependent on Ca concentrations in the water rather than in their food, indicating that Ca is obtained mainly from the water. However, the relative importance of dietary and waterborne Ca has not been quantified, and the importance of dietary Ca under Ca-deficient conditions has not been studied.

The homeostasis and regulation of Ca in crustaceans during the molt cycle have been studied (Wheatly 1999; Wheatly et al. 2002; Ahearn et al. 2004). The currently accepted model for unidirectional influx of waterborne Ca^{2+} in crustaceans proposes that Ca^{2+} enters the apical membrane of epithelial cells via carrier-mediated facilitated diffusion or simple diffusion through Ca^{2+} channels and crosses the basolateral membrane by active transport, which employs plasma membrane Ca^{2+} adenosine triphosphatase and Na^+/Ca^{2+} exchanger (Wheatly et al. 2002). However, the regulation of Ca in crustaceans (especially

* Corresponding author: wwang@ust.hk

zooplankters) in different Ca environments has not been explored. It was found that the fish *Sparus auratus* can react rapidly to changes in Ca availability in the environment by employing endocrine control of Ca metabolism using both hyper- and hypocalcemic hormones (Abbink et al. 2004); larvae of freshwater tilapia (*Oreochromis mossambicus*) increased the influx and reduced the efflux of Ca in a low-Ca environment (0.8 mg L^{-1}) to maintain plasma Ca levels (Chou et al. 2002). However, in *Daphnia*, Alstad et al. (1999) found that even when the ambient Ca concentration was very low (0.5 mg L^{-1}), body Ca was still lost without efficient reclamation during molting. Whether or not *Daphnia* have a greater ability to incorporate Ca from water and/or food in low-Ca environments has not been established.

In this study, we used the radioisotope ^{45}Ca as a tracer to quantify the dissolved uptake rate of Ca from water, the assimilation efficiency (AE) of Ca from the diet, and the efflux of body Ca in juvenile (4-d) and adult (10-d) *D. magna* cultured in low (0.5 mg L^{-1})– and high (50 mg L^{-1})–Ca environments. Our objective was to study the regulation of Ca influx (both from water and food) and efflux in different Ca environments by daphnids; a second objective was to study the differences between juvenile and adult daphnids in terms of their ability to regulate Ca. The well-established biokinetic model (Guan and Wang 2006) was also employed to evaluate the relative importance of water and food as the source of Ca and to predict the Ca content in daphnids under different conditions.

Methods

Organisms, medium, and radioisotope—A clone of *D. magna*, which has been successfully cultured in our laboratory for more than 7 yr, was used in this study. Freshwater obtained from an unpolluted brook on the campus of The Hong Kong University of Science and Technology (Kowloon, Hong Kong, China) was used for maintaining the daphnid stock after being filtered with GF/C membrane. The Ca concentration of the water was around 20 mg L^{-1}. Ten milliliters of water was allocated to each daphnid and the water was changed every 2 d. A green alga, *Chlamydomonas reinhardtii*, was fed to the daphnids daily at a concentration of 10^5 cells mL^{-1} (2.73 mg carbon [C] L^{-1}), and half that concentration was fed to the daphnids that were less than 4 d old. The algae were cultured in WC medium (Guillard and Lorenzen 1972) until mid-log phase, centrifuged, resuspended in filtered brook water, and stored at 4°C in a refrigerator before use. The light–dark cycle was 14 : 10 h light : dark for the culturing of daphnids and algae, and the temperature was 23.5°C.

In order to control the Ca concentrations, synthetic freshwater Elendt M7 (Samel et al. 1999) was used instead of brook water for culturing daphnids in the experiments for quantifying the biokinetic parameters. All other conditions remained the same as for the culturing stock daphnids, except where noted. In the dissolved uptake experiments, simplified M7 medium (SM7, containing only $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, $NaNO_3$, $NaHCO_3$, Na_2SiO_3, H_3BO_3, and KCl, and without ethylenediaminetetraacetic acid [EDTA], trace metals, and vitamins) was used as the exposure medium. The Ca concentration of the media was modified by the addition of $CaCl_2$, and the pH was adjusted to 8.0 ± 0.2 using HCl and NaOH solutions. The actual Ca concentrations were measured at the beginning and end of all experiments, and the average values for the low- and high-Ca treatments were 0.57 ± 0.05 mg L^{-1} and 51.9 ± 2.2 mg L^{-1}, respectively. For a specific treatment, nominal Ca concentrations in the medium for culturing daphnids, algae, and resuspending algae were the same. In the present study, juvenile daphnids were 4 d old and adult daphnids were 10 d old.

The radioactive isotope ^{45}Ca (440.10 MBq mg^{-1}) was used as a tracer in biokinetic measurements. The isotope was purchased from PerkinElmer and dissolved in water.

Dissolved uptake and dietary assimilation of Ca—The uptake rate of Ca from the dissolved phase and the AE of dietary Ca were both quantified in four separate experiments: juvenile daphnids (4 d) cultured in a low-Ca (0.5 mg L^{-1}) environment (LJ) or a high-Ca (50 mg L^{-1}) environment (HJ) and adult daphnids (10 d) cultured in a low-Ca environment (LA) or a high-Ca environment (HA). To prepare daphnids for each experiment, neonates (<24 h old) of the stock daphnids were cultured for 4 d (juvenile) or 10 d (adult) in M7 medium including one of the two Ca concentrations. Before the biokinetic measurements, the daphnids were evacuated in M7 medium for approximately 2 h to minimize the effects of feces during the dissolved uptake experiments or to starve the daphnids for the AE experiments. A portion of the daphnids (~40 individuals) was collected for Ca content measurement, and the remaining daphnids were used for the dissolved uptake or AE experiments. An effort was made to choose daphnids of similar size for the experiments.

The dissolved uptake rate of Ca for each group of daphnids was quantified at five Ca concentrations (i.e., 0.5, 2, 10, 50, and 200 mg L^{-}; measured Ca concentration: 0.52 ± 0.04, 2.0 ± 0.1, 11.1 ± 0.2, 53.7 ± 0.6, and 212 ± 4 mg L^{-1}, respectively) in the SM7 medium, and three replicates were used for each concentration. After their guts were evacuated, the daphnids were rinsed twice with SM7 medium (the Ca concentrations were the same as the medium in which they were cultured) to remove EDTA, and then 20 (adult) or 24 (juvenile) individuals were allocated into each high-density polyethylene beaker containing 100 mL SM7 media spiked with ^{45}Ca for the 6-h dissolved uptake experiment. No molting occurred during the experiments. For the five Ca concentrations, the addition of ^{45}Ca comprised 148, 148, 185, 370, and 740 kBq L^{-1} (adult) or 148, 370, 592, 740, and 1480 kBq L^{-1} (juvenile), respectively. Compared with the stable Ca, the amounts of ^{45}Ca were negligible. After 1, 2, 4, and 6 h, five (adult) or six (juvenile) individuals were chosen for radioactivity measurements. To avoid the effects of radioisotope weakly adsorbed by daphnids or contained in the water covered under the carapaces, the animals were placed in a series of three beakers containing approximately 100 mL nonradioactive M7 media of corresponding Ca

concentrations and were allowed to swim for 1–2 min. At the beginning and end of the exposure, a 0.5-mL water sample was taken for radioactivity measurement, and the average of the readings was used for the calculations. The newly incorporated Ca into the daphnids (C, mg g dry wt^{-1}) was calculated as

$$C = (A \times C_w)/(A_w \times 1000) \quad (1)$$

where A is the radioactivity in the daphnids (counts per min g dry wt^{-1}) (CPM), A_w is the radioactivity in the water (CPM mL^{-1}), and C_w is the Ca concentration in the water (mg L^{-1}). The dissolved uptake rate (J, mg g dry wt^{-1} h^{-1}) of Ca was calculated as the time-weighted average of C following the equation

$$J = \sum C_i \Big/ \sum t_i \quad (2)$$

where t_i is the time of exposure (h) and C_i is the concentration of the newly incorporated Ca after t_i (mg g dry wt^{-1}).

The AE experiment for each group of daphnids was conducted at five food abundance levels (i.e., 2×10^3, 5×10^3, 10^4, 2×10^4, and 10^5 cells mL^{-1}), and three replicates were used for each food concentration. To prepare radiolabeled food for the low-Ca experiments (LJ, LA), early log-phase *C. reinhardtii* were centrifuged and resuspended into modified WC medium (without EDTA, copper, and zinc), and grown for 3–4 d under continuous lighting. The Ca concentration in the medium was 0.5 mg L^{-1} of the stable Ca with 7.4 MBq L^{-1} ($\sim$17 μg L^{-1}) ^{45}Ca. The initial cell density was 2×10^5 cells mL^{-1}, and after the 3–4 d of growth, the cells divided three to four times and the algae were collected, centrifuged, and resuspended into M7 medium. The centrifugation and resuspension procedures were repeated once more to remove the weakly bound ^{45}Ca, and then the labeled algae were immediately used in the AE experiment. To label the algae with enough ^{45}Ca for the high-Ca experiments (HJ, HA), the procedures of labeling were different from those used for the low-Ca experiments. In detail, the early log-phase algae cultured in WC medium (10 mg Ca L^{-1}) were centrifuged and resuspended into a Ca-free modified WC medium at an initial cell density of 2×10^5 cells mL^{-1} and ^{45}Ca was spiked at a concentration of 11.1 MBq L^{-1} ($\sim$25 μg L^{-1}). After 45–60 h of continuous lighting, 50 mg L^{-1} of stable Ca was added, and after 3 d of growth, the algae were harvested following the same procedures described above.

After 2 h of starvation, 25–36 daphnids were placed in each high-density–polyethylene beaker containing 10 mL M7 medium for each individual. The radiolabeled algae were then fed to the animals for 15 min in the dark. At the end of the pulse feeding, five (adult) or six (juvenile) daphnids were picked out and placed in 100 mL of M7 medium of the corresponding Ca concentration and allowed to swim for 1 min, and the radioactivity was quantified. The remained daphnids were depurated in nonradioactive M7 medium with nonradioactive food added at corresponding cell densities for 24–39 h. Water and food were renewed at 3, 7, 12, 18, 24, and 30 h, and five (adult) or six (juvenile) daphnids were collected at each renewal time for radioactivity assay, except at 18 h and 30 h.

Efflux of Ca—The efflux of Ca from *D. magna* was investigated in both juvenile (4–7-d) and adult (10–14-d) daphnids, which were raised in media with low (0.5 mg L^{-1}) or high (50 mg L^{-1}) Ca concentrations. For the experiments on juvenile *D. magna*, newly born (<24-h) daphnids were cultured in SM7 medium spiked with ^{45}Ca for 4 d for radiolabeling. For the experiments on adult daphnids, neonates (<24 h) were cultured in M7 medium for 4 d, and they were exposed to ^{45}Ca in SM7 medium for the following 6 d to reach the age of 10 d. The addition of ^{45}Ca was at the rate of 185 kBq L^{-1} for the low-Ca treatments and the rate of 1.85 MBq L^{-1} for the high-Ca treatments. In order to reduce the use of ^{45}Ca, 5 mL of water was allocated to each daphnid during the radiolabeling period; the green algae *C. reinhardtii* was added as food at a concentration of 10^5 cells mL^{-1} twice per day. Hessen and Rukke (2000) reported that the intermolt period was less than 2 d for juvenile *D. magna* and gradually increased to almost 5 d for reproducing adults. Therefore, it is reasonable to assume that both the juvenile and adult daphnids were evenly labeled with ^{45}Ca after the 4–6 d of exposure.

The radiolabeled daphnids were transferred into nonradioactive M7 medium containing 10^5 cells mL^{-1} of *C. reinhardtii* for 1–2 h to depurate their feces, which might have contained ^{45}Ca. After that, the daphnids were depurated in M7 medium of corresponding Ca concentrations for 3–4 d to investigate the efflux of Ca. Three replicates were used in each treatment. In order to have enough radioactivity in the water samples during the efflux period, 6 mL of water was allocated to each individual (instead of 10 mL). For the experiments on juvenile and adult daphnids, different sampling procedures were employed. For the juvenile daphnids, four individuals were randomly picked out at the time of 0, 0.25, 0.5, 1, 1.5, 2, and 3 d for radioactivity measurements. The volume of water was adjusted according to the number of daphnids remaining. To investigate the contribution of excretion and molting to efflux, 5 mL of medium and the generated molts were collected for radioactivity assays at each sampling time. The radioactivity of the adult daphnids was only measured at the beginning and end of the efflux experiments, and no animal was sampled for measurements during the depuration period. Water samples, molts, and newly born neonates were collected for measurement on days 0.5, 1, 1.5, 2, 3, and 4. The radioactivity in adult daphnids was calculated by the mass balance method. For both experiments, water was renewed at each time point and food was added twice a day at a concentration of 10^5 cells mL^{-1}. A linear regression between the natural log value of the percentage of retained ^{45}Ca and the depuration time (d) was conducted, and the absolute values of the slopes were calculated as the efflux rate constants (k_e).

Chemical and radioactivity analysis—The Ca concentrations of the daphnid samples and water samples were measured using flame AAS (PerkinElmer AAnalyst 800) after proper dilution to the concentration range of 0.4–

Table 1. The specific Ca content (% dry wt), predicted steady-state Ca content of *Daphnia magna*, and the relative importance of water (S_w) and food (S_f) as the source of Ca for *D. magna* (*see* the modeling in Discussion section).*

	LJ	HJ	LA	HA
Ca content (% dry wt)	1.99±0.01	4.71±0.21	1.77±0.26	3.82±0.28
Predicted (% dry wt)	1.43	4.72	0.46	1.83
S_w (%)	97.9	99.9	97.1	99.9
S_f (%)	2.1	0.1	2.9	0.1

* The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}). The second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Values are mean ± standard deviation (n = 3).

4 mg L^{-1}. The final samples contained approximately 2% HNO_3 and 1% lanthanum in order to avoid interference. To prepare the daphnid samples, the animals were rinsed with nanopure water and dried at 80°C for 1–2 d and were then weighed to the nearest 10 μg. The dried daphnids were then ashed at 500°C for 2 h and dissolved with 2% HNO_3 after cooling.

The radioactivity in the samples was measured using a Beckman LS-6500 Liquid Scintillation Counter. The samples were counted for 3 min, or longer if necessary, to ensure propagated counting errors of less than 5%. To prepare the daphnid samples, the daphnids were transferred into a 7-mL scintillation vial and digested with 0.5 mL 30% HNO_3 at 80°C for 6 h. Thereafter, 5 mL of scintillation cocktail (PerkinElmer) was added and the mixtures were well mixed by mechanical shaking. After 8 h, the radioactivity was measured using the scintillation counter. The 5-mL water samples were measured in 20-mL scintillation vials, combined with 15-mL scintillation cocktail following the same method. The acid-quenching effect was quantified and used for correction of the data.

Results

Ca content—The specific Ca content of the daphnids was significantly affected by the Ca level of the medium and also by the age of the daphnids ($p < 0.05$, two-way ANOVA; Table 1). Daphnids cultured in the 50 mg Ca L^{-1} medium had 2.2 to 2.4 times higher Ca content than did the daphnids in low-Ca (0.5 mg L^{-1}) medium, and juvenile daphnids had 10–20% higher Ca content than adults.

Uptake of dissolved Ca—The dissolved uptake rates of Ca at the five Ca concentrations (0.5–200 mg L^{-1}) in *D. magna* are described by the Michaelis–Menten kinetic equation (Fig. 1):

$$J = \frac{J_{\max} \times C_w}{K_m + C_w} \quad (3)$$

The maximum dissolved uptake rates of Ca (J_{max}, mg g dry wt^{-1} h^{-1}) of the juvenile daphnids ranged from 3.2 to 4.1 mg g dry wt^{-1} h^{-1}, much higher than those of the adult daphnids (i.e., 1.5–1.6 mg g dry wt^{-1} h^{-1}; Table 2). Two-way ANOVA showed that the age (juvenile or adult) of the daphnids had significant effects on J_{max} ($p < 0.01$), while the effects of Ca content in the daphnids were not significant ($p > 0.05$). The Michaelis–Menten constants (also called the half-saturation constants, K_m, mg L^{-1}) were 2.2–2.5 mg L^{-1} for juvenile daphnids and 5.6–8.8 mg L^{-1} for adults (Table 2). Although lower K_m values were observed in juvenile daphnids, two-way ANOVA indicated that there were no significant effects from either age or Ca level ($p > 0.05$). These results indicated that the uptake of Ca was greater in juvenile than in adult daphnids, while it was comparable among daphnids reared in different Ca environments.

AEs of Ca—The pattern of Ca depuration over time differed among the different groups (LJ, HJ, LA, and HA) of *D. magna*; however, most rapid depuration happened during the first 12 h in all groups, after which only trivial release of ingested Ca occurred in most cases (Fig. 2). Therefore, the AEs of Ca were calculated as the percentage of retained ^{45}Ca after 12 h of depuration following the mass balance method (Yu and Wang 2002), and they are summarized in Table 3. Three-way ANOVA showed that the AEs of Ca were significantly affected by the Ca level ($p < 0.001$) and age ($p < 0.001$) of the daphnids and the food abundance level ($p < 0.001$). There were significant interactions between the life stage and the food concentration ($p < 0.001$). Increasing the food concentration decreased the AE of Ca; the relationship between Ca AE and the food carbon concentration can be described with an exponential equation (Fig. 3). Most of the AEs were in the range of 20–40% when the food concentrations were 2×10^3 to 10^4 cells mL^{-1} and below 15% when the food concentrations were above 2×10^4 cells mL^{-1}. For daphnids of the same age, the AEs of Ca were generally higher in daphnids cultured in low-Ca than in high-Ca environments. Adult daphnids had higher Ca AE than did juveniles when food was scarce (2–5 $\times$ 10^3 cells mL^{-1}); however, when food was relatively abundant, the adults had lower Ca AE than did the juveniles. This indicates that adult daphnids are more sensitive to changes in food concentration than are juvenile daphnids in terms of Ca AE.

Efflux of Ca—The Ca was lost rapidly from the daphnids during the 3–4 d of depuration (Fig. 4A). Daphnids cultured in the low-Ca environment consistently retained a higher percentage of ^{45}Ca than did the daphnids cultured in the high-Ca environment. After 1.5–2 d, approximately 10–20% and 1–2% of ^{45}Ca was retained in the daphnids cultured in the low-Ca and high-Ca environments, respectively. Two-way ANOVA showed that Ca level had a significant effect on the k_e of Ca ($p < 0.001$), while the effect of age was not significant ($p = 0.05$). The k_e of the daphnids cultured in the low-Ca and high-Ca environments were 0.83–0.86 d^{-1} and 1.59–1.98 d^{-1}, respectively (Table 4). Excretion into water (60–83%) was the dominant route of Ca loss for both juvenile and adult daphnids cultured in low- and/or high-Ca environments,

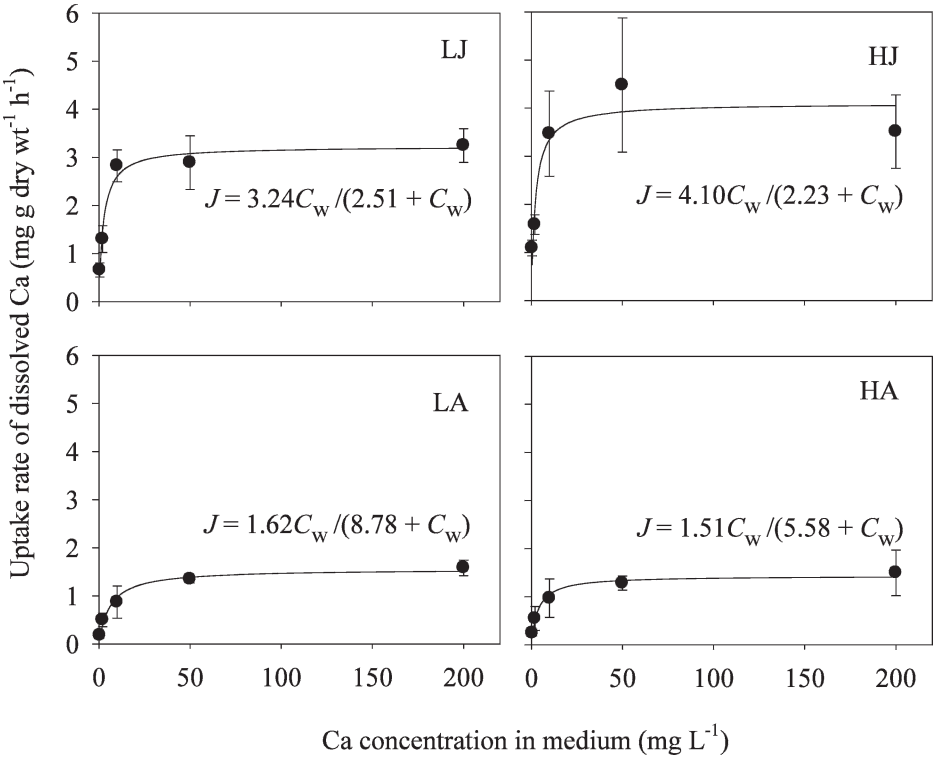

Fig. 1. The relationship between the influx rate of Ca (J) by *Daphnia magna* and Ca concentrations in the medium (C_w) described by the Michaelis–Menten equation. The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}); the second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Error bars represent standard deviations (n = 3).

and molting (17–40%) was also an important route for Ca efflux (Fig. 4B). For adult daphnids, Ca required for reproduction represented a very small portion of the Ca loss (i.e., around 1%; Fig. 4B). The relative contribution of excretion and molting was comparable between juvenile and adult daphnids in both low- and high-Ca environments. The dominance of excretion was more substantial for the daphnids cultured in the low-Ca environment. When the loss of ^{45}Ca through molting was higher, the excretion was also relatively higher (Fig. 5).

Discussion

Dissolved uptake—Juvenile daphnids had 2.1–4.4 times higher weight-specific influx rates of Ca (mg g dry wt^{-1} h^{-1}) than adult daphnids in the medium with the same Ca concentration, indicating the greater ability of juveniles to cope with Ca limitation. However, it was reported that neonate *D. galeata* had lower survival rates than adults in a Ca-deficient medium (Rukke 2002), possibly because the higher influx rate of Ca in the juvenile daphnids was not

Table 2. Dissolved uptake rate (J) of Ca by *Daphnia magna* in the SM7 medium of different Ca concentrations (0.5–200 mg L^{-1}) and the calculated maximum dissolved uptake rate (J_{max}) and half-saturation concentration (K_m).*

	LJ	HJ	LA	HA
Ca in medium (mg L^{-1})		J (mg g dry wt^{-1} h^{-1})		
0.5	0.66±0.15	1.09±0.16	0.18±0.05	0.25±0.06
2	1.30±0.28	1.58±0.20	0.50±0.14	0.55±0.25
10	2.83±0.33	3.47±0.88	0.87±0.33	0.97±0.40
50	2.89±0.56	4.48±1.40	1.35±0.08	1.29±0.15
200	3.25±0.35	3.51±0.76	1.58±0.16	1.50±0.47
J_{max} (mg g dry wt^{-1} h^{-1})	3.24±0.39^a	4.10±0.55^a	1.62±0.18^b	1.51±0.39^b
K_m (mg L^{-1})	2.51±0.83^a	2.23±0.52^a	8.78±7.62^a	5.58±4.37^a

* The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}). The second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Values are mean ± standard deviation (n = 3). Values of J_{max} and K_m with the same superscripted lowercase letters do not significantly differ ($p > 0.05$, t-test).

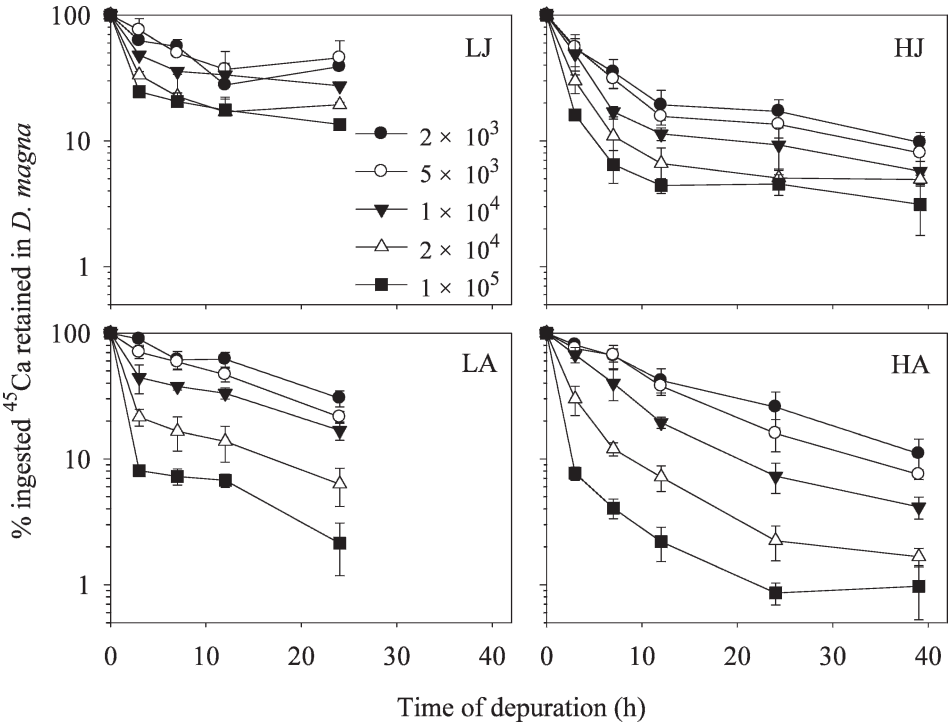

Fig. 2. The retention of Ca in *Daphnia magna* during the 24–36-h depuration following the 15-min pulse feeding. The food (*Chlamydomonas reinhardtii*) concentrations were the same during the pulse feeding and depuration periods and ranged from 2×10^3 to 10^5 cells mL^{-1}. The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}); the second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Error bars represent standard deviations ($n = 3$).

enough to compensate for their higher Ca demand. This is supported by the finding that juvenile daphnids molt more frequently (Hessen and Rukke 2000) and have higher Ca contents (Alstad et al. 1999). Similarly, 3-d-old larvae fish (*O. mossambicus*) have faster responses and greater capability for compensation than do newly hatched larvae when they confront a low-Ca (0.8 mg L^{-1}) environment (Chou et al. 2002). The sea bream *Sparus auratus* can react rapidly to changes in Ca availability in the environment by employing endocrine control of its Ca metabolism using both hyper- and hypocalcemic hormones (Abbink et al. 2004). However, such regulation was not observed in daphnids of the present study (i.e., daphnids cultured in low- and high-Ca environments had comparable influx rates of Ca in media with the same Ca concentration).

Table 3. Assimilation efficiency (AE) of Ca in the food (*Chlamydomonas reinhardtii*) by *Daphnia magna* at different food concentrations (2×10^3–10^5 cells mL^{-1}).*

Food density ($\times 10^3$ cells mL^{-1})	AE of Ca (%)			
	LJ	HJ	LA	HA
2	27.7 ± 0.2^{ab}	19.3 ± 5.9^{a}	62.0 ± 8.2^{c}	42.0 ± 10.1^{bc}
5	37.0 ± 14.4^{ab}	15.6 ± 1.0^{a}	46.8 ± 5.9^{b}	37.9 ± 4.4^{b}
10	33.4 ± 8.1^{a}	11.4 ± 1.3^{b}	33.3 ± 3.4^{a}	19.5 ± 2.0^{c}
20	17.0 ± 5.2^{a}	6.6 ± 2.2^{b}	13.8 ± 4.4^{ab}	7.2 ± 1.7^{b}
100	17.6 ± 3.9^{a}	4.4 ± 0.6^{b}	6.8 ± 0.8^{c}	2.2 ± 0.7^{d}

* The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}). The second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Values are mean ± standard deviation ($n = 3$). Values with the same superscripted lowercase letters do not significantly differ ($p > 0.05$, *t*-test).

Our results cannot explain the juvenile bottleneck for Ca, as suggested by Hessen et al. (2000), because juvenile daphnids had higher weight-specific influx rates of Ca than did adults in waters of the same Ca concentrations, which indicates that juveniles should be more capable of extracting Ca from water. However, our data do not necessarily refute this juvenile bottleneck hypothesis, because we do not know the Ca demands of daphnids, except that juvenile daphnids had slightly higher specific Ca content. The ability of daphnids to cope with Ca deficiency should be dependent on the difference between Ca demand and supply, as well as on the relevant physiological differences between daphnids of different life stages.

The K_m (half-saturation constants) of Ca in *D. magna* in the present study (2.1–8.8 mg L^{-1}) were comparable to those of previous studies in a wide range of freshwater organisms: for example, 5.2 mg L^{-1} in the postmolt crayfish *Austropotamobius pallipes* (Greenaway 1974), 12 mg L^{-1} in the amphipod *Gammarus pulex* (Wright 1979), 12 mg L^{-1} in the snail *Lymnaea stagnalis* (Greenaway 1971), and 2.5–5.6 mg L^{-1} in the fishes *Fundulus heteroclitus* and *Oncorhynchus mykiss* (Patrick et al. 1997). It seems that the K_m of Ca for freshwater organisms falls

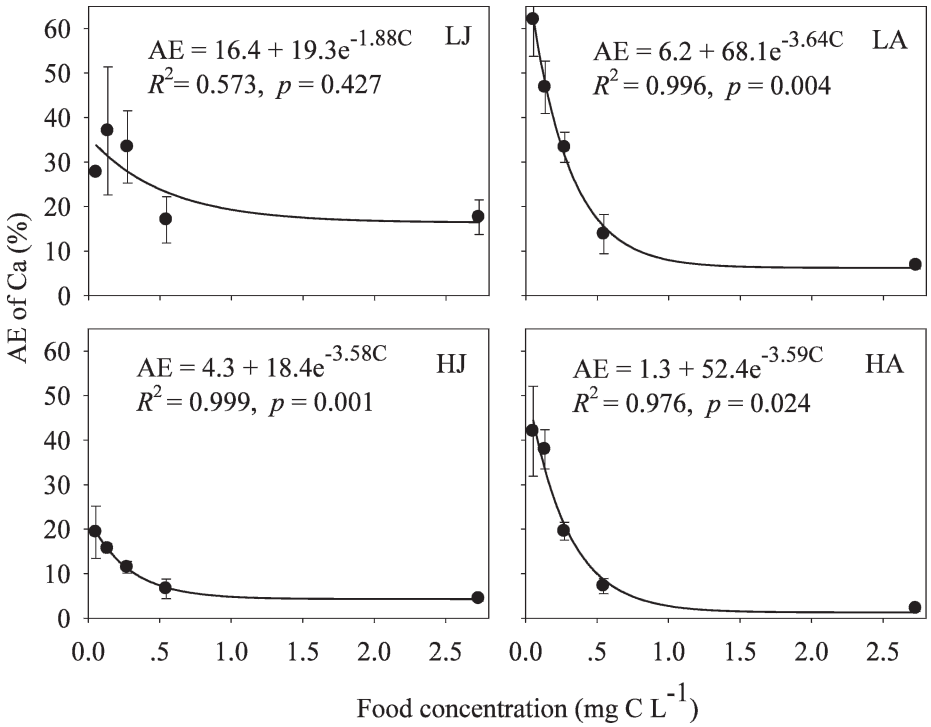

Fig. 3. The relationship between assimilation efficiency (AE) of Ca from food (*Chlamydomonas reinhardtii*) and the food carbon concentration described by an exponential equation. The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}); the second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Error bars represent standard deviations ($n = 3$).

within a rather narrow range (i.e., 2–12 mg L^{-1}). The global mean concentration of freshwater Ca is 15 mg L^{-1} (Markert et al. 1997), indicating that a considerable proportion of freshwaters have a Ca concentration that is near or below the Ca requirements of *D. magna* and possibly other aquatic organisms (Jeziorski et al. 2008). Therefore, Ca limitation may often occur in freshwater ecosystems. For example, a survey of 342 large Norwegian lakes showed that the median Ca concentration was 2.4 mg L^{-1} (Hessen et al. 1995*b*); a study of 23 Ontario (Canada) lakes showed that all of the Ca concentrations were below 2.6 mg L^{-1} (mean, 1.7 mg L^{-1}) (Keller et al. 2001). Moreover, the Ca concentration may decline in these areas as a result of recovery from acidification after reduction in the emissions of SO_2 (Skjelkvåle et al. 1998; Keller et al. 2001; Jeziorski et al. 2008). Hessen et al. (2000) found that *D. magna* suffered from subsaturation of Ca (judged from growth and egg production) when the Ca concentration was lower than 10 mg L^{-1}. A saturation Ca concentration for the calcification of the exoskeleton in *D. magna* of between 5.2 and 10 mg L^{-1} was recorded (Alstad et al. 1999).

The influx rates of Ca in the present study in juvenile and adult daphnids were 0.66–1.58 and 0.18–0.55 mg g dry wt^{-1} h^{-1}, respectively, when the ambient Ca concentration was 0.5–2 mg L^{-1}, and 2.83–4.48 and 0.87–1.58 mg g dry wt^{-1} h^{-1} when the ambient Ca concentration was 10–200 mg L^{-1}. In our experiments, it was difficult to distinguish the newly molted (postmolt) daphnids from the intermolt ones, and the influx rates were quantified with several daphnids rather than one individual. Therefore, the influx rates should be considered as average values of the molting cycle. Theses values are quite high when compared with the available data obtained in intermolt freshwater crustaceans (i.e., 0.56 μg g^{-1} h^{-1} in the crayfish *A. pallipes* [Greenaway 1972] when the ambient Ca was 24 mg L^{-1} and 11.5 μg g^{-1} h^{-1} in the amphipod *G. pulex* [Wright 1979] when the ambient Ca was 8 mg L^{-1}). The influx rates in the present study were even higher than those quantified in the postmolt crayfish *Orconectes virilis* (i.e., 0.29 mg g dry wt^{-1} h^{-1} [90.4 μg g fresh wt^{-1} h^{-1}, a fresh wt : dry wt of 3.26 was used (Angeler et al. 2001) when the ambient Ca was 2.8 mg L^{-1} (Malley 1980)]). The high influx rate of Ca is vital for the rapid calcification of postmolt *Daphnia*, because *Daphnia* do not have an efficient mechanism for reclaiming Ca from their old exoskeletons (Alstad et al. 1999), which is common in other crustaceans (solubilized exoskeleton Ca that is transferred and stored for later use in calcifying the new exoskeleton is used by many species) (Ahearn et al. 2004).

Dietary assimilation—Elevating the food concentration leads to a higher ingestion rate of food particles and in turn may result in a shorter gut residence time (Evers and Kooijman 1989), which is probably the main reason for the decreased Ca AEs with increasing food concentrations. In zooplankton, there exists an incipient limiting food concentration (ILC), at which the zooplankton achieve

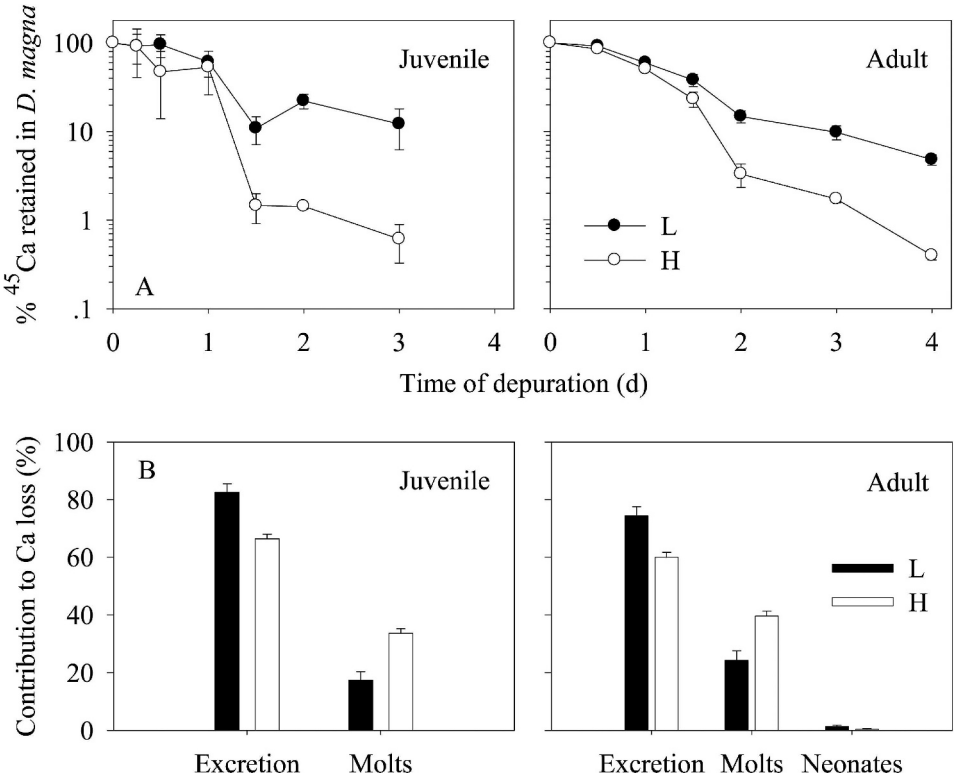

Fig. 4. (A) The retention of ^{45}Ca in juvenile (4–7-d) and adult (10–14-d) *Daphnia magna* during the 3–4-d depuration at low (L, 0.5 mg L^{-1})– and high (H, 50 mg L^{-1})–Ca concentrations following the 4–6-d exposure to combined sources (both dietary and waterborne) of ^{45}Ca at each corresponding Ca concentrations and (B) the relative contributions of excretion, molting, and reproduction to the total efflux of ^{45}Ca during the 3-d depuration. Error bars represent standard deviations ($n = 3$).

the maximum ingestion rate and, accordingly, a minimum AE. The ILC for 2.7-mm (adult) *D. magna* feeding on *C. reinhardtii* was reported to be 0.5 mg C L^{-1} (Porter et al. 1982), which was consistent with our observation: the decrease in AE was trivial when the food concentration was higher than 0.5 mg C L^{-1} (2×10^4 cells mL^{-1}) (Fig. 3). It was found that the ILC of the cladoceran *Sida crystallina* increased with body size (Downing and Peters 1980), indicating that adult animals have higher ILCs than do juveniles, which could explain the greater drop in Ca AE in adult daphnids at high food concentrations. When the food concentration was higher than 0.5 mg C L^{-1} (2×10^4 cells mL^{-1}), the AEs of Ca were relatively comparable between juvenile and adult daphnids. When the food concentration was lower than 0.13 mg C L^{-1} (5×10^3 cells mL^{-1}), the adult daphnids had much higher Ca AE (Fig. 3), indicating the important role played by longer gut and gut residence times of larger daphnids (Evers and Kooijman 1989) in the food-deficient environment. The contribution of dietary assimilation of Ca was almost negligible when compared to aqueous uptake (*see* later discussion); therefore, the regulation of dietary assimilation according to body or ambient Ca levels seems unimportant. However, it was found that daphnids cultured in higher Ca media had lower Ca AEs. This may be due to competition of Ca in the gut environment, which could be dependent on the ambient Ca level, because crustaceans can incorporate Ca through drinking water (Zanotto and Wheatly 2003). The inhibition of dietary assimilation by Ca was also observed for cadmium and zinc (Tan and Wang 2008).

Table 4. Efflux rate constant (k_e) and biological retention half-life ($t_{1/2}$) of Ca in *Daphnia magna*.

	LJ	HJ	LA	HA
k_e (d^{-1})	0.83 ± 0.14^a	1.98 ± 0.24^b	0.86 ± 0.04^a	1.59 ± 0.00^c
$t_{1/2}$ (d)	0.85 ± 0.16^a	0.35 ± 0.04^b	0.81 ± 0.04^a	0.44 ± 0.00^c

* The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}). The second letter represents the age of daphnids: J = juvenile (4 d) and A = adult (10 d). Values are mean ± standard deviation ($n = 3$). Values with the same superscripted lowercase letters do not significantly differ ($p > 0.05$, *t*-test).

Efflux—The k_es of Ca (0.86–1.98 d^{-1}) in *D. magna* were quite high when compared with those of other metals, metalloids (0.01–0.48 d^{-1}), and phosphorus (0.10–0.30 d^{-1}) quantified in previous studies (He and Wang 2007; Tsui and Wang 2007). There has been no study on the k_e of Ca in *Daphnia*. However, we can make rough estimates from the data of Alstad et al. (1999). At the temperature 18°C, 90% of Ca was lost during one molting. Assuming that the molting frequency is 5 d, the estimated k_e is $(\ln 100 - \ln 10)/5 = 0.46\ d^{-1}$. This probably

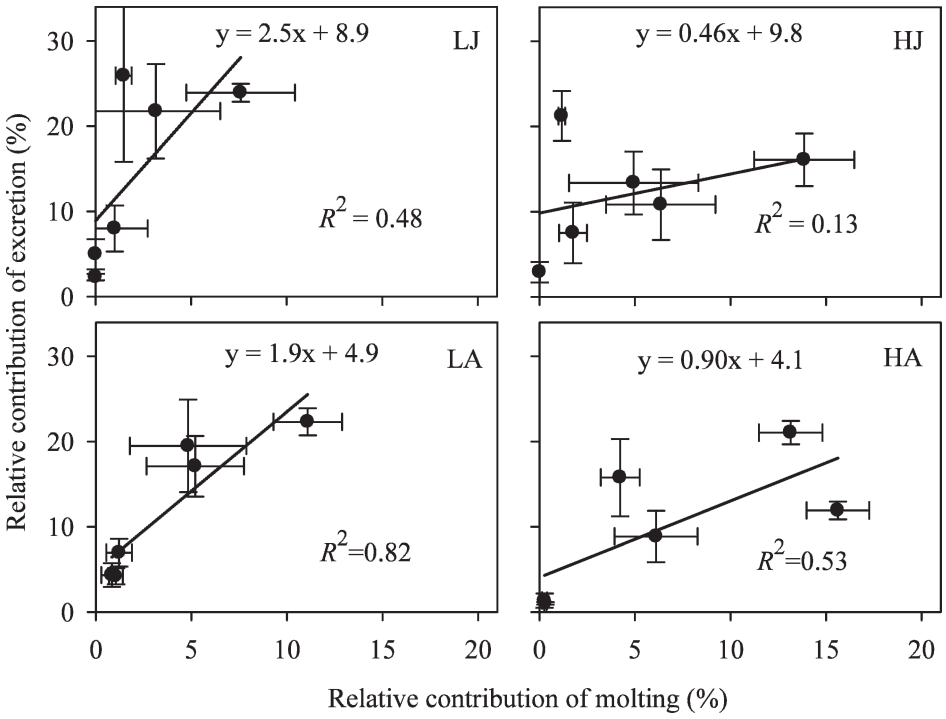

Fig. 5. The relationship between the relative contribution of excretion and molting to the total efflux of Ca of *Daphnia magna* described by a linear regression. The first letter in each treatment represents the Ca level in which the daphnids were cultured from birth: L = low (0.5 mg Ca L^{-1}) and H = high (50 mg Ca L^{-1}); the second letter represents the age of daphnids: J = juvenile (4–7 d) and A = adult (10–14 d). Error bars represent standard deviations (n = 3).

underestimates the k_e because it only considers the efflux caused by molting (and the associated leakage into water), while molting-independent excretion is not included. In addition, the k_es were 1.8–2.4 times higher in the high-Ca environment than in the low-Ca environment, indicating that Ca is regulated in daphnids facing different ambient Ca concentrations. However, the regulation ability was very limited when there was no significant regulation in Ca influx.

The term excretion in this study was practically defined as the release of Ca into the water, which may be associated with molting process or may be attributable to basic metabolism. Excretion played a more important role than molting during efflux (by 1.5–4.7-fold measure), especially in the low-Ca environment. This is to some extent in agreement with the observation of Alstad et al. (1999) that during molting, 40% of the body Ca was contained with the shed molt, while 50% was lost into the water. In the high-Ca environment, molting contributed more to the efflux of Ca. It was reported that the molting frequency of daphnids was independent of ambient Ca level (Hessen et al. 2000); therefore, the higher contribution of molting was possibly not due to the higher frequency of molting but rather to the higher Ca content in the molt (in the low-Ca environment, the carapace is not fully calcified). A positive correlation between molting and excretion in Ca efflux was observed (Fig. 5), possibly as a result of the substantial leakage of Ca into water during the molting (Alstad et al. 1999).

The small effect of reproduction on the loss of Ca (compared to molting and excretion) indicates the low maternal transfer of Ca. We speculate that the exoskeletons of newly released neonates were not fully calcified and that they thus had very low Ca content. This was indirectly indicated by the finding that Ca was mainly distributed in the eggshell but not in the embryos (which would develop into neonates) of the resting eggs of *D. magna*, as observed by X-ray microscopy (Kawasaki et al. 2004), although there is a difference between the embryos of resting eggs and neonates produced by parthenogenesis. It was also directly determined that newborn daphnids had lower Ca content than did young and mature daphnids (1.75% vs. 3.71–6.89%); and in terms of Ca, one newborn daphnid contains 2.7–4.3% of the Ca in one mature daphnid (Baudouin and Ravera 1972). However, it should be noted that the "newborn" daphnids in this field study were judged by size, and the animals might have incorporated Ca from water before being sampled.

Modeling—The biokinetic model (Eq. 4) has been successfully utilized in predicting trace metal concentrations in a wide range of aquatic animals, with most predictions lying within a twofold measure of the observed values (Luoma and Rainbow 2005):

$$C_{ss} = (J + \mathrm{AE} \times \mathrm{IR} \times C_f)/(k_e + g) \qquad (4)$$

where C_{ss} is the steady-state metal concentration in the animals (mg g dry wt^{-1}), IR is the weight-specific ingestion rate (g dry wt g dry wt^{-1} d^{-1}), C_f is the specific metal content of the diet (mg g dry wt^{-1}), and g is the growth rate

of the animal (d^{-1}). The relative importance of dissolved uptake as the source of Ca (S_w) can be calculated by the following equation:

$$S_w = J/(J + AE \times IR \times C_f) \times 100\% \quad (5)$$

The relative importance of dietary uptake (S_f) is calculated as ($1 - S_w$). Assuming that IR = 0.96 g dry wt g dry wt^{-1} d^{-1} (in the food-abundant environment) and that C_f = 2 mg g dry wt^{-1}, the S_w and S_f for juvenile and adult daphnids in the low- and high-Ca environments were calculated (Table 1). Based on the model, dissolved uptake from water is the dominant source of Ca for daphnids (>97%). For the daphnids in the low-Ca environment, the contribution of dietary assimilation was 2–3%, and it was below 1% in daphnids in the high-Ca environment. Assuming g = 0.3 and 0.1 d^{-1} for juvenile and adult daphnids (Q.-G. Tan and W.-X. Wang unpubl.), respectively, the predicted steady-state Ca contents of daphnids are also summarized in Table 1. The predicted Ca contents in the juvenile daphnids matched well with the measured values (1.0–1.4 times), although the Ca contents in the adult daphnids were underestimated by a 2.1-fold to 3.8-fold measure.

To conclude, the influx rate of Ca from the water is very high in *D. magna* and is accompanied by high efflux and short half-retention time. Ca in the water is the dominant source of Ca for *D. magna* reared in different Ca environments and with different food levels. The contribution of dietary Ca is relatively more important in the low-Ca environment. Daphnids returned 60–85% of extracted Ca back into the water, and 15–40% was temporarily lost as molts. Daphnids in the low-Ca environment had higher AEs for Ca from food and lower efflux rate constants (k_e) for Ca than did daphnids in the high-Ca environment. However, the maximum influx rate (J_{max}) and half-saturation concentration (K_m) of Ca were comparable between daphnids living in different Ca environments. The uptake of aqueous Ca was responsible for the higher Ca content of daphnids in the high-Ca environment, which was downregulated by higher efflux. Juvenile daphnids had higher J_{max} and lower K_m of Ca than did adult daphnids, indicating that juvenile daphnids had a greater ability to incorporate Ca from water, which does not explain the juvenile bottleneck in the low-Ca environment. Compared with adult daphnids, juvenile daphnids were less sensitive to food concentrations in terms of Ca AEs, and they had comparable (in low-Ca medium) or lower (in high-Ca medium) k_e.

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