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EFFECTS OF EXTERNAL CALCIUM CONCENTRATIONS ON CALCIUM UPTAKE IN FRESHWATER SNAIL *Planorbarius corneus* L.

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ABSTRACT

Calcium content of water is of fundamental importance for freshwater snails since it is correlated with growth rates, egg fertility and fecundity. In this study, calcium uptake from the external medium (tap water) in two groups of freshwater snail *Planorbarius corneus* L. was compared: 200 control snails permanently maintained in tap water, and 200 experimental snails exposed to solutions with high calcium concentrations (214–2137 mg/L) for six weeks before the measurements. The results showed higher uptake in control snails (0.8 mg Ca^{2+} per snail/day) than in experimental snails (0.4 mg Ca^{2+} per snail per day), $P<0.001$. Measurements of Ca^{2+} in shells and tissues showed a higher calcium content in experimental snails than in control snails, both in the shells ($P<0.02$) and in the tissue ($P<0.05$). Lower shell/tissue calcium ratio and lower organic mass loss by ashing showed that experimental snails stored 1.5 times more Ca^{2+} in soft tissues compared with control snails. Empty dry shells of the control snails accounted for 27% of the total body mass, and in the experimental snails, shell mass was 34% of the body mass. The results suggest that the lower uptake by experimental snails was caused by previously accumulated high quantities of calcium in shells and soft tissues. These findings enhance our understanding of calcium homeostasis in freshwater gastropods and indicate their adaptability to environments with fluctuating calcium levels.

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INTRODUCTION

Many studies documented the importance of calcium content of water for freshwater snails and the correlation between calcium content and population densities for most pulmonates (Dussart, 1976, 1979; Harrison et al., 1970; Macan, 1950; Russel-Hunter, 1964; Thomas et al., 1974; Ebanks et al., 2010). Variability in freshwater calcium content influences species' physiology, reproduction, distribution and abundance (Dillon, 2000; Russel-Hunter, 1964). Increases in available calcium are correlated with higher growth rates, egg fertility and fecundity (Mackie and Flippance, 1983; Madsen, 1987; Thomas et al., 1974). Increased calcium availability induces stronger, larger, heavier and thicker shells, which have significant ecological and evolutionary implications as inducible defence from predators (Brodersen and Madsen, 2003; Bukowski and Auld, 2014; Rundle et al., 2004). Low environmental calcium can have deleterious consequences in different freshwater gastropods. It increases metabolic demand and can cause reduced growth and survival, as well as decreases the ability to adapt to novel stressors (Dalesman et al., 2011), causes reduced growth and survival of juveniles (Hunter, 1990), and can have a highly significant effect on locomotion and respiration (Dalesman and Lukowiak, 2010). Investigations on chronic metal toxicity to freshwater pulmonate snails show that growth inhibition is associated with Ca^{2+} uptake inhibition (Brix et al., 2017; Cremazy et al., 2018; Rainbow, 2018).

The surface epithelium of snails is highly and selectively permeable to the calcium ion, even more permeable than to sodium ion (Greenway, 1971a). Snails use calcium primarily for the formation of their shells and they absorb calcium from the external aquatic medium (Van der Borght, 1962, 1963; Greenway, 1971a; Thomas and Lough, 1974). In a long-term experiment with *Lymnaea stagnalis* in water with a trace of ^{45}Ca, Van der Borght and Van Puymbroeck (1966) showed that about 80% of the calcium body content of the snails is absorbed from the water and 20% from the food. Experiments by Thomas and Lough (1974) showed that freshwater snails can lower the external calcium concentrations to values of less than 1 mg/L, in some cases to 0.5 mg/L, and that the uptake increases with increasing calcium concentrations in the medium.

In this study on freshwater snail *Planorbarius corneus* (Linnaeus, 1758), the aims were to investigate:

1. whether the experimental snails previously exposed to high calcium concentrations would absorb less calcium from the external medium than control snails grown permanently in tap water;
2. whether the experimental snails would have more calcium content in their shells and tissues, stored from the previous exposure to high calcium concentrations, compared to control snails;
3. whether the calcium uptake by snails would increase with increased calcium concentrations in external water;
4. and whether the calcium content in experimental snails would increase if previously exposed to increased calcium concentrations.

MATERIALS AND METHODS

From the laboratory culture, 200 snails of equal size (~10 mm) were selected as control snails to be compared with 200 snails of the same size previously exposed to seven different solutions containing high concentrations of $CaCl_2$ or $CaBr_2$ for six weeks. Calcium concentrations to which these snails were exposed were: 214, 434, 534, 869, 1069, 1732 and 2137 mg/L. Ten snails were placed into each 1000 ml covered glass beaker (100 ml per snail). There were also three blank samples, beakers with 1000 ml of water without snails. The experiment was carried out for one week and the calcium concentrations in the water were measured at the start and end of the experiment using a Varian Techtron AA5 atomic absorption spectrometer. The experimental water was dechlorinated tap water with daily fluctuations of calcium concentrations ranging from 39 mg/L to 160 mg/L due to several sources of water in the municipal water supply. Water samples with snails were set and Ca^{2+} was measured over three weeks, one, two or three samples a day, which is why the calcium concentrations at the start were not equal in all samples. The snails were fed daily on washed organic lettuce leaves ad libitum and held under natural photoperiod at 23 ± 1°C, pH 6.8–7.3 and O_2 concentrations of 8 mg/L. At the end of the experiment, the calcium concentrations in the shells and tissues of several groups of control and experimental snails were measured for comparison of shell/tissue Ca^{2+} ratio and mass loss by ashing. The samples were prepared by the nitric acid digestion method. Tissue samples were evaporated, rinsed with distilled water and dried. The shells were dried in Petri dishes at 105 °C for two hours. Each sample was then placed in a washed, dried, burned and weighed platinum crucible, after which it was weighed and ashed at 500 °C to a constant weight. The ash was treated with 2 ml nitric acid (MERCK Darmstadt), digested and dried twice and dissolved in 0.1N hydrochloric acid (MERCK Darmstadt). Calcium concentrations in the samples were then determined in the spectrophotometer. The statistical evaluation of differences in changes of Ca^{2+} concentrations in water within one week was performed using a nonparametric Mann-Whitney test, since the Shapiro-Wilk test showed that the distribution of the samples was not normal and the F test showed that the variances were unequal. After the Shapiro-Wilk test showed a normal distribution in the measurements of Ca^{2+} content in snails, the statistical evaluation of differences in Ca^{2+} content between the control and experimental treatments was calculated by Student's t-test.

RESULTS

After one week, calcium concentrations significantly decreased in all water samples with control snails (P<0.001, Table 1). Calcium uptake by the snails ranged from 12% to 95%, i.e. 52% on average (each control sample represented the mean of two measurements). In water samples with experimental snails, calcium concentrations absorbed by snails were from 0% (in two cases) to 48% (in one case), i.e. 26% on average. Since no loss of calcium by adsorption on the glass was detected in three blank samples, it was confirmed that the calcium was absorbed by the snails. In neither group, the amount of calcium uptake did not depend on Ca^{2+} concentrations in external water. Mean calcium absorption in the controls from 1L of water in one week was 56 mg, which is 5.6 mg per snail. Mean calcium absorption in water samples with experimental snails was significantly lower at 27 mg, 2.7 mg per snail (P<0.001). The mean mass of 10 mm long control snails was 260.45 ± 2.09 mg and the mean mass of experimental snails of the same size was 290.40 ± 2.31 mg. Based on this, the mean weekly uptake of calcium would be 2% of the total body mass for control snails and 0.9% of the total body mass for experimental snails.

Table 1. Changes in calcium concentrations over 7 days in tap water with snails: K_1-K_{20}, control snails; E_1-E_{20} experimental snails previously exposed to noted high calcium concentrations (214 - 2137 mg/L) for six weeks; P < 0.001

Sample \ Ca^{2+} mg/L		1st day	7th day	Absorbed mg/L	Absorbed (%)
Blanks tap water	1	83.81	84.28	0	0
	2	82.52	83.79	0	0
	3	82.79	84.72	0	0
K_1		73.97	64.86	9.11	12
K_2		95.89	65.17	30.72	32
K_3		102.23	46.74	55.49	54
K_4		101.20	26.09	75.11	74
K_5		101.89	34.51	67.38	66
K_6		118.35	88.85	29.50	25
K_7		117.72	76.76	40.96	35
K_8		118.35	60.01	58.34	49
K_9		79.96	24.24	55.72	70
K_{10}		79.77	37.39	42.38	53
K_{11}		111.07	62.39	48.68	44
K_{12}		62.40	4.66	57.74	92
K_{13}		63.0	2.92	60.08	95
K_{14}		160.87	51.48	109.39	68
K_{15}		163.04	44.70	118.34	72
K_{16}		164.13	50.00	114.13	69
K_{17}		122.15	74.40	47.75	39
K_{18}		111.99	69.90	42.09	38
K_{19}		111.39	90.12	21.27	19
K_{20}		78.35	38.57	39.78	51
$\overline{X}$		106.89 ± 30.33	50.69 ± 27.71	56.20 ± 29.35	52.85 ± 22.87

Continued. Table 1

Sample	Ca^{2+} mg/L	1st day	7th day	Absorbed mg/L	Absorbed (%)
E_1	214	39.30	20.22	19.08	48.00
E_2	214	127.61	56.09	71.52	56.00
E_3	434	116.93	103.96	12.97	11.00
E_4	434	162.68	143.22	19.46	12.00
E_5	434	97.87	73.50	24.37	25.00
E_6	434	109.79	83.68	26.11	24.00
E_7	534	39.06	25.22	13.84	35.00
E_8	534	65.95	41.20	24.75	37.00
E_9	534	112.38	65.80	46.58	4100
E_{10}	869	121.19	123.00	0.00	0.00
E_{11}	869	97.68	85.60	12.08	12.00
E_{12}	869	120.41	121.72	0.00	0.00
E_{13}	1069	130.55	57.70	72.85	56.00
E_{14}	1069	39.18	21.23	17.95	5.00
E_{15}	1069	65.97	35.22	30.75	46.00
E_{16}	1069	162.65	147.03	15.62	10.00
E_{17}	1732	120.09	107.92	12.20	10.00
E_{18}	1732	104.89	62.09	42.80	41.00
E_{19}	1732	103.01	65.55	37.46	36.00
E_{20}	2137	162.68	114.41	48.27	29.00
$\overline{X}$		104.99 ± 38.55	77.72 ± 39.70	27.43 ± 20.39	26.70 ± 18.30

Measuring Ca^{2+} in the shells and tissue of several groups of control and experimental snails (Table 2) showed that calcium content in control snails was lower than in experimental snails ($P<0.02$ for the shells and $P<0.05$ for the tissue). The ratio shells/tissue calcium content was higher in control snails, which pointed to the higher calcium content in experimental snail tissue. The difference in mass loss by ashing between control and experimental snails also suggested higher calcium concentrations in experimental snail tissue. Empty dry shells of the control snails had an average mass of 70 ± 1.54 mg, which was 27% of the total body weight, and experimental snails had an average shell mass of 99.89±2.04 mg (1.4 times heavier). Measuring Ca^{2+} content in the groups of experimental snails showed no dependence on the Ca^{2+} concentrations of previous exposure.

Table 2. Calcium content in shells and tissue, and mass loss by ashing in snails: K_1-K_5 control snails; E_1-E_9 experimental snails previously exposed to noted high calcium concentrations mg/L for six weeks; $P < 0.02$ (shells Ca^{2+} content); $P < 0.05$ (tissue Ca^{2+} content)

Ca^{2+} mg/g / Sample	Shells	Tissue	Ratio shells/tissue	Mass loss by ashing (%)	
				Shells	Tissue
K_1	264.83	20.10	13.2	5.01	90.54
K_2	274.15	20.67	13.3	1.82	90.36
K_3	255.90	18.42	13.9	1.83	91.43
K_4	254.95	17.38	14.7	2.45	91.12
K_5	217.59	11.20	19.4	2.08	91.96
$\bar{X}$	253.484 ± 21.52	17.554 ± 3.79	14.9 ± 2.58	2.638 ± 1.35	91.082 ± 0.65
E_1 214 mg/L	334.44	29.25	11.4	2.71	88.91
E_2 214 mg/L	261.27	21.26	12.3	3.01	89.25
E_3 434 mg/L	301.55	27.77	10.8	2.77	88.68
E_4 434 mg/L	282.79	2252	12.6	5.06	88.48
E_5 869 mg/L	282.28	23.30	12.1	2.39	89.37
E_6 1069 mg/L	294.49	37.19	7.9	2.09	81.15
E_7 1069 mg/L	28936	25.21	11.5	3.88	87.48
E_8 1732 mg/L	293.80	24.61	11.9	2.47	83.12
E_9 1732 mg/L	303.10	31.32	9.7	2.15	83.37
$\bar{X}$	293.676 ± 19.78	26.937 ± 5.05	11.133 ± 1.49	2.948 ± 0.96	86.645 ± 3.18

DISCUSSION

Aquatic gastropods absorb calcium from the external aquatic medium and use it for the formation of their shells (Greenway, 1971a,b; Van der Borght, 1962, 1963). Laboratory and field investigations in different snail species showed a correlation between environmental calcium and formation of the shell, for example heavier shells at higher Ca^{2+} concentrations in four Planorbids (Madsen, 1987) increased shell diameter and weight with increase in calcium concentrations in Planorbid *Biomphalaria sudanica* (Brodersen and Madsen, 2003), stronger shells at high Ca^{2+} concentrations (Rundle et al., 2004), heavier, thicker and larger shells with Ca^{2+} availability (Bukowski and Auld, 2014). Surface epithelium of snails is highly and selectively permeable to calcium ions and has high-affinity mechanisms for calcium uptake (Greenway, 1971a; Thomas and Lough, 1974). Calcium uptake by snails from external media which contain less than 20 mg/L Ca^{2+} is an active process (movement against electrochemical gradient), but at Ca^{2+} concentrations above 20 mg/L minimal energy is required (Greenway, 1971a; Thomas and Lough, 1974). Greenway (1971a) showed that the net calcium uptake from artificial water containing 40 mg/L Ca^{2+} by individual *Lymnaea stagnalis* was 20 mg/L in twelve days (≈ 1.7 mg/day per snail), and the rate of uptake by different snails or by the same snail at different times was extremely variable. Our experiments also showed that the uptake by control snails was very variable. Among 20 beakers with 10 equally sized snails each, it varied from 12% to 95%, and the maximum absorption in our experiments was 11.8 mg/L per snail in 7 days, which is also 1.7 mg/L/day per snail. Experiments with another pulmonate snail *Biomphalaria glabrata* showed that the values for the uptake rates were considerably higher than those for *Lymnaea stagnalis* reported by Greenway (1971a), and that the net calcium uptake by snails increased with an increase in calcium concentrations in the medium (Thomas and Lough, 1974). Grossel and Brix (2009) reported that in 1 g *Lymnea stagnalis* snails, Ca^{2+} influx rate was 750 nmol g^{-1} h^{-1} (0.03 mg Ca g^{-1} h^{-1}) but in juvenile snails net Ca^{2+} uptake rates were 7000 – 8000 nmol g^{-1} h^{-1} (Brix et al., 2012), which is 0.28 – 0.32 mg Ca g^{-1} h^{-1}. In our experiments,

the calculated minimum Ca^{2+} influx rate was 0.02 mg Ca g^{-1} h^{-1}, and the maximum influx rate was 0.27 mg g^{-1} h^{-1}. Differences depending on Ca^{2+} concentrations in external water were not noticed. Greenway (1971a) reports that calcium uptake is not related to the external calcium in a linear manner. Differences in uptake rates could be explained by differences in temperature, phase of growth and natality rates (Thomas and Lough, 1974). In- and out-fluxes of Ca^{2+} in freshwater gastropods were studied by Van der Borght (1963) in *Lymnaea stagnalis*. Use of tracer ^{45}Ca showed that the absorbed Ca was accumulated at the inside of the outer border of the shell, that Ca uptake starts immediately after placing the snails into the solution at the same time with a net loss of Ca from the animals, and that net uptake begins after few hours even when only 2.5 mg of Ca is present in the solution. Another experiment with ^{45}Ca (Greenway, 1971b) on *Lymnaea stagnalis* shows that calcium absorbed from the medium by freshwater snails moves between three calcium compartments: the shell, the blood and the fresh tissue, and the major calcium-containing compartment is the shell. The absorbed calcium appears first in the blood and then in the shell and other tissues. During calcium loss in calcium-free water, there is a net movement of calcium from the shell to the blood. Freshwater gastropods store calcium in soft tissues (Franchini and Ottaviani, 1993; Haley and Gibson, 1971). Investigation on *Helisoma duryi* showed that significantly higher numbers of calcium spherules were stored in the snails grown in the lowest (10 mg/L) and highest (320 to 640 mg/L) calcium concentrations compared to intermediate concentrations. This suggests that the active mechanism is more active at extreme calcium concentrations and that passive absorption at higher concentrations (above 160 mg/L) accounts for the increased storage (Haley and Gibson, 1971). In our study, experimental snails previously exposed to high calcium concentrations (214 – 1732 mg/L) stored 1.5 times more Ca^{2+} in soft tissues compared to control snails, which is suggested by lower shell/tissue ratio and lower mass loss by ashing. Measurements of Ca^{2+} in the shells and tissue of the control and experimental snails showed that calcium content in all control snails was lower than in experimental snails (P<0.02 for the shells and P<0.05 for the tissue). The empty dry shells of the control snails had an average mass of 70 mg, which was 27% of the total body weight, and the experimental snails had a 30% heavier average shell mass, 100 mg or 34% of the body weight. In comparison, the shell accounts for 25% of the total snail mass in control *Lymnaea stagnalis* (Grosell and Brix, 2009). In this study, the calcium absorption from tap water in experimental snails previously exposed to solutions with very high calcium concentrations (27.43 mg Ca^{2+}) was more than half the amount of control snails (56.70 mg Ca^{2+}).

CONCLUSIONS

From the results, it could be concluded that the shells and tissues of the experimental snails were saturated with previously accumulated calcium, which caused a lower uptake compared to the control snails. The study showed that snails exposed to very high Ca^{2+} concentrations in external medium stored significantly more calcium in shells and tissues compared to snails exposed to average water concentrations. The study also confirmed that the rate of Ca^{2+} uptake by snails was extremely variable. The amount of absorbed calcium in all groups did not depend on Ca^{2+} concentrations in external water. The calcium content in the experimental snails did not depend on previous exposure concentrations.

UČINAK KONCENTRACIJE KALCIJA IZ VANJSKOG MEDIJA NA UNOS KALCIJA U SLATKOVODNOG PUŽA *Planorbarius corneus* L.

SAŽETAK

Sadržaj kalcija u vodi od životne je važnosti za slatkovodne puževe jer je povezan s brzinom rasta, uspjehom oplodnje jaja, obimom i brzinom razmnožavanja i rastom kućice. U ovom radu uspoređena je apsorpcija kalcija iz vanjskog medija (vodovodne vode) u dvije grupe slatkovodnog puža *Planorbarius corneus* L.: 200 kontrolnih puževa stalno držanih u vodovodnoj vodi i 200 pokusnih puževa izloženih otopinama visokih koncentracija kalcija (214–2137 mg/L) tijekom šest tjedana prije mjerenja. Rezultati su pokazali veću apsorpciju kod kontrolnih puževa (0.8 mg Ca^{2+} po pužu/danu) nego u pokusnih puževa (0.4 mg Ca^{2+} po pužu/danu), P<0.001. Mjerenje Ca^{2+} u kućicama i tkivima pokazalo je viši sadržaj kalcija u pokusnih, nego u kontrolnih puževa, i u kućicama (P<0.02) i u tkivima (P<0.05). Niži omjer kalcija između kućica i tkiva, i manji gubitak mase žarenjem pokazali su da su pokusni puževi pohranili 1.5 puta više kalcija u tkivima od kontrolnih. Suhe prazne kućice kontrolnih puževa iznosile su 27% od ukupne tjelesne mase, a u pokusnih puževa masa kućica bila je 34% ukupne mase puža. Rezultati ukazuju da je niža apsorpcija u pokusnih puževa bila prouzročena prije akumuliranom velikom količinom kalcija u tkivima i kućicama.

Ključne riječi: apsorpcija kalcija, sadržaj kalcija, učinak vanjske visoke koncentracije

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