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Accumulation of ^{45}Ca in the Freshwater Unionids *Anodonta anatina* and *Unio tumidus*, as Influenced by Water Hardness, Protons, and Aluminum

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ABSTRACT Previous studies have revealed that freshwater bivalves subjected to acid stress suffer from severe electrolyte imbalance. A typical initial reaction is a transitory increase in concentration of Ca in the hemolymph during the first few days of exposure to such stress. Although this calcium obviously originates from the $CaCO_3$ reserves in the clam, a possible ambient origin has never been ruled out. In the present study the uptake of Ca was studied under both circumneutral and acid conditions: two freshwater unionids, *Anodonta anatina* and *Unio tumidus*, were exposed to a bathing medium that contained ^{45}Ca for a period of 24 hrs. In order to study the effect of the ambient Ca concentration on the efficiency of uptake of Ca, two media with different Ca concentrations (35 and 3.5 mg Ca/l; 4×10^6 cpm ^{45}Ca/l and 4×10^5 cpm ^{45}Ca/l, respectively) were used. The role of the ingested food as a source of Ca was evaluated by feeding the clams with algae labeled with ^{45}Ca. After the clams had been exposed for 24 hrs to ^{45}Ca, the accumulation of ^{45}Ca in the soft parts, including hemolymph, calcium concretions and glochidial larvae, was measured. Low pH (4–4.5) significantly decreased the uptake of ^{45}Ca from the medium, whereas the addition of aluminum (900 μg/l) had only a minor effect on the uptake of ^{45}Ca. The sites of accumulation of ^{45}Ca under acidified conditions did not change: in all cases highest values for ^{45}Ca concentration were measured in the gills, where the calcium concretions sequestered a large part of the ^{45}Ca. A 90% decrease in the ambient Ca concentration caused a similar decrease in the uptake of ^{45}Ca. Clams were able to utilize both dietary and waterborne Ca.

Freshwater unionid clams inhabit waters that range from the extremely soft (less than 1 mg Ca/l) to the moderately hard (Mackie and Flippance, '83, Pynnönen, '90). Their hemolymph is one of the most dilute of all aquatic animals (Potts, '54); 99.71% of its volume is reported to consist of water (Malley et al., '88). To preserve electrolyte balance the clams have to concentrate ions from the surrounding medium and simultaneously minimize the loss of ions to the medium. For aquatic animals with calcified shells, such as molluscs and crustaceans, a constant supply of Ca is of a great importance. In addition to being used for shell growth, Ca in the freshwater bivalves is also used to build the shells of the developing glochidial larvae which are bred in the water channels of the outer gill demibranches. Since shells of the mature larvae from a single *Anodonta* can contain as much as 400 mg of calcium (Silverman et al., '87), a large amount of calcium is lost by the females each reproductive season. Therefore, to guarantee normal reproduction, an adequate uptake of Ca is required and even a minor disturbance in such uptake, caused, for example, by freshwater acidification, could lead to impaired reproduction and gradual disappearance of unionids from acid waters.

Species of *Unio* and *Anodonta* have been found in waters in different trophic states and with different ionic composition (Agrell, '49), and the weight of their shells depends on the type of water in which they live. In general, a positive correlation can be found between the calcium content of the individual and the ambient Ca concentration (Hinch et al., '89), but this is not always the case. Thus, interspecific variations may occur with respect to the efficiency of uptake of Ca.

Previous studies have demonstrated that acid stress severely disturbs the normal electrolyte balance of these freshwater bivalves (Pynnö-

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A decrease in the hemolymph pH was compensated by an increase in the Ca concentration of the hemolymph, the latter probably originating from the large reserves of $CaCO_3$ in the shell and mantle. The validity of this hypothesis is challenged by the fact that severe acidosis tends to cause thickening rather than erosion of the shells of *Anodonta* (Machado et al., '88). Under ammonium or metabolic acid stress, Ca is not liberated from the calcified concretions of the gills (Silverman et al., '83, Pynnönen, '90). It has been observed in crustaceans (Malley and Chang, '85) and in fish (Reader and Morris, '88) that the uptake of Ca is suppressed by an acid medium. Kat [illegible] suggested that unionids do not occur in waters with high $[H^+]$ (< pH 4.5) because such conditions would lead to rapid rates of dissolution of shells and would make it difficult for the unionids to concentrate calcium carbonate. However, the effect of low pH on the uptake of Ca in these clams has never been demonstrated.

Gills of freshwater molluscs are generally considered to be the main site for the exchange of ions, but in addition, the uptake can apparently take place over the entire body surface. The Ca concentrated from the ambient medium or from ingested food particles is sequestered in the shell and in the calcified concretions of the gills; these concretions make up 25–50% of the dry weight of the gills of these bivalves (Pynnönen et al., '87). The origin of the calcium has never been studied in detail, but it has been shown that only a small amount of the calcium in the larval shell is derived from the bathing medium (Silverman et al., '87). The developing glochidia in the water channels of the gills draw their calcium for their shells directly from the reserves of $CaCO_3$ that are present in the gills in the form of calcium concretions.

This report describes the uptake of Ca in two species of freshwater unionids in water of various pH values and hardness. In addition, the effect of aluminum, a metal often present at elevated concentrations in acidified waters, was studied. The purpose of this study was to evaluate the sensitivity of the species to severe acid stress and to reveal possible differences between the efficiency of the uptake of calcium in soft and hard water. Since the origin of calcium in bivalve molluscs has not been studied in detail, the uptake of Ca from the two most obvious sources, namely, water and diet, was measured, and the distribution of dietary and waterborne calcium in the various organs was compared.

MATERIALS AND METHODS

Animals

Freshwater clams (*Unio tumidus* L., *Anodonta anatina*, L.) were collected in September 1988 from ditches in the Maarsseveen lake district near Utrecht. The concentration of calcium in water in the sampling area varied between 50 and 53 mg Ca/l and the pH varied between 7.5 and 7.6. The shell length of the animals used in the experiments varied between 6.5 cm and 8.3 cm (*U. tumidus*) and between 6.0 cm and 8.0 cm (*A. anatina*). Both females and males were used. During the experimental period specimens of *U. tumidus* were carrying ripening eggs and sperm, and those of *A. anodonta* carried mature glochidia.

In the laboratory, the clams were kept in 150-l aquaria supplied with a sandy substrate, plants, small fish, and snails. The animals were kept in running tapwater (for composition, see Table 1) at a temperature of 13 ± 1°C and under natural illumination. Clams were fed weekly on *Chlorella*.

Exposures

Uptake of Ca by the specimens of *A. anatina* and *U. tumidus* over a period of 24 h was followed in a bathing medium that contained ^{45}Ca ions ($CaCl_2$ in H_2O, specific activity 10 mCi/mg Ca; Amersham, the Netherlands). Experiments were performed in 500-ml glass vials at a temperature of 19°C. Clams were exposed in individual baths. Continuous aeration was maintained and the bathing medium was changed after each exposure. Before the onset of each exposure and immediately after termination of the exposure period of 24 hrs, 100-μl samples were taken from the bathing medium to check the specific activity. The same sampling procedure was also performed on vials without clams to monitor the possible adsorption of ^{45}Ca ions to the surface of the glass.

Experiments were performed in circumneutral tapwater (for chemical composition see Table 1) and in tapwater that has been acidified by the addition of sulfuric acid until the solution had a pH of 4–4.5. In both cases the medium contained 35 mg Ca/l and 3,833 – 5,303 × 10^3 cpm ^{45}Ca/l. During and after the experiments the pH of the labeled water in the vial was not checked, but in the experiments performed under identical conditions in unlabeled medium, the pH at the end of a 24-hr period was found to be between pH 5.5 and 6.0. In order to study the effect of the external

TABLE 1. Chemical composition of the experimental water

Element	Hard	Soft
Ca milligram/liter (mmol/l)	35 (0.88)	4.6 (0.12)
Na milligram/liter (mmol/l)	11 (0.47)	0.3 (0.01)
Cl milligram/liter (mmol/l)	15 (0.45)	NM
K milligram/liter (mmol/l)	0.7 (0.02)	ND
Mg milligram/liter (mmol/l)	3.5 (0.15)	1.0 (0.04
Fe milligram/liter (mmol/l)	0.1 (0.02)	0.3 (0.06)
Al milligram/liter (mmol/l)	ND	ND
pH milligram/liter (mmol/l)	8.0–8.3	6.6–7.0

Levels of Cl^- were measured with a chloride titrator, the pH was measured with a KCl electrode, and the other elements were quantitated with a flame atomic absorption spectrophotometer for hard water and with an inductively coupled plasma atomic emission spectrophotometer for soft water.
ND, not detected; NM, not measured.

Ca on the accumulation of calcium, tapwater was diluted with demineralized water at a ratio of 1:10. This medium contained 3.5 mg Ca/l and 301 – 545 × 10^3 cpm ^{45}Ca/l. The concentration of Ca in the exposure medium was measured with an atomic absorption spectrophotometer (Varian spectrAA 10). Specimens of both species were studied under each set of conditions. In addition, the uptake of ^{45}Ca by *U. tumidus* was studied in an acidified, aluminum-supplemented (total concentration of aluminum, 900 μg/l) medium which contained 35 mg Ca/l and 3,648 – 4,017 × 10^3 cpm/l. Aluminum was added in the form of $AlCl_3$. No experiments were performed in soft, acid water, since it was not possible to stabilize the pH in static systems with low buffering capacity.

All clams were kept in an aquarium without substratum and were fed for three days prior to the experiments. A sudden exposure to acidic or to extremely soft water may cause a violent avoidance reaction that leads to a valve closure. Therefore, before measurements of the uptake of ^{45}Ca uptake, clams were acclimated to the new conditions for 24 hrs.

For the experiments with algae, *Chlorella* was grown for 72 hrs in a medium that contained ^{45}Ca-label (10 × 10^4 cpm/ml). The suspension of algae was centrifuged and the pellet was resuspended in tapwater after several washes with tapwater. An algal suspension was added to the exposure vials that contained 500 ml of tapwater (35 mg Ca/l). To 4 vials, only the labeled algae suspension was added, and to 4 vials the suspension was added and the water was supplemented with ^{45}Ca to a nominal activity of 3,000 × 10^3 cpm/ml. Samples were taken from all vials and their radioactivity was measured. The amounts of ^{45}Ca in the exposure baths were as follows (mean ± SEM):

—label in water only, 3,815 (±996) cpm/ml (n = 6)
—label in *Chlorella,* 218 (±26) cpm/ml (n = 4
—label in water and *Chlorella,* 3,157 (±142) cpm/ml (n = 4)

In order to study the affinity for calcium of calcium concretions in the gills of these bivalves, one sample of *A. anatina* and one of *U. tumidus* were exposed simultaneously in one 500-ml vial for 24 hrs. A total of 8 specimens, 4 of each species, were exposed. The activity of the bath varied between 1630 and 2123 cpm/ml.

Sampling

At the end of the exposure period of 24 hrs hemolymph samples of 0.5 ml were taken from the pericardium of the clams by use of a 1-ml syringe fitted with a 22-gauge needle. Samples of 0.02–0.10 g (dry weight) were cut from the middle region of the gills (both left and right demibranches), mantle, hepatopancreas and kidney. In order to monitor the distribution of ^{45}Ca in the organs, several samples were taken from various parts of the gills, mantle and hepatopancreas-kidney and glochidia. When glochidia were found a sample was taken for the analysis of ^{45}Ca. For the measurements of the ^{45}Ca-activity in the calcium concretions of the gills, small pieces from the gill demibranches of the clams were sampled and calcium concretions were isolated by the method described earlier by Pynnönen et al. (1987).

All samples of tissue and calcium concretions were lyophilized for 24 hrs to a constant dry weight. Weighed and wetted (with distilled water) samples of tissue and calcium concretions were dissolved in 0.5 ml of Lumasolve (Lumac b.v. the Netherlands, no. 1042) and acidified with a few drops of concentrated HCl to complete the disintegration. *Chlorella* cells were dissolved in Lumasolve before samples of water from the feeding experiments were analyzed.

Measurements

Four ml of Emulsifier-SAFE (Packard Instrument Company, Inc., the Netherlands) scintillation fluid were added to the samples of decomposed tissue and to the samples of water and hemolymph. In order to diminish the chemiluminescence, samples were left in the dark for 10–12 hrs prior to the quantitation of radioactivity. Radioactivity in samples of water and hemolymph

TABLE 2. Radioactivity of ^{45}Ca (cpm/ml) in the bathing medium prior to the onset of the exposure and the decrease in radioactivity (cpm/ml and as % of the original activity) during the 24 hrs of exposure

Exposure	^{45}Ca at start cpm/ml mean (±SEM)	Decrease in activity cpm/ml (%)	[Ca] (mg/l)	Number of exposures
Ut, pH 8	4030 (±60)	584.2 (14.5%)	35	6
Aa, pH 8	3820 (±406)	517.0 (13.5%)	35	6
Ut, pH 8	440 (±53)	199.0 (45.2%)	3.5	4
Aa, pH 8	370 (±41)	139.3 (37.6%)	3.5	4
Ut, pH 4–4.5	4030 (±52)	266.2 (6.6%)	35	6
Aa, pH 4–4.5	4020 (±95)	445.0 (11.1%)	35	6
Ut, pH 4–4.5, (900 µg Al/l)	3840 (±12)	46.5 (1.2%)	35	6
Aa + Ut, pH 8	1837 (±96)	97.3 (5.3%)	35	8

Means ± SEM are given. Ut, *U. tumidus;* Aa, *A. anatina.*

as quantitated (without prior decomposition) after scintillation fluid had been added. Within the week of sampling radioactivity was counted using a liquid scintillation counter. Radioactive decay over a period of one week was of minor importance (about 3%), and, thus, no correction was required. Each sample was counted twice and an external standard was used to correction for quenching.

Statistical analysis

The differences in the rates of uptake of ^{45}Ca in various organs of *U. tumidus* and *A. anatina* were tested for significance by Student's t-test or by the Cochran method when sample variances were unequal. Linear regression analysis was used to determine the correlation between Ca concentration in hemolymph and gills, and between Ca in hemolymph and glochidia.

RESULTS

Uptake of Ca by various organs

The amount of ^{45}Ca accumulated in the organs during a 24-hr exposure to ^{45}Ca-labeled medium was taken as a measure of the efficiency of uptake of calcium. The radioactivity of the bathing medium was checked before the onset and after termination of the exposure (Table 2), but it could not be used as the sole parameter for quantitation of uptake because the amount adsorbed onto the shell surface was not known. In the vials without a clam, the radioactivity of the medium was not markedly decreased over the period of 24 hrs, indicating that there had been no marked adsorption of Ca to the surface of the glass. Since the distribution of the label over the organs was found to be homogeneous (Table 3), the concentrations measured in the small samples of tissue were taken as an accurate representation of the accumulation of ^{45}Ca in the organs.

In the circumneutral (pH 8), hard medium the distribution of ^{45}Ca among the organs was very similar in the two species (Fig. 1, white columns): gills accumulated most of the ^{45}Ca, mantle, hepatopancreas and kidney accumulated lesser amounts which were almost equal to one another. The concentration in the organs was generally about 3 to 4 times lower than that in the gills. The concentration of ^{45}Ca in the hemolymph was somewhat higher in *U. tumidus,* both in hard (35 mg Ca/l) and in soft (3.5 mg Ca/l)

TABLE 3. The homogeneity of the distribution of ^{45}Ca in the various organs of U. tumidus *and* A. anatina

	Hemol. cpm/ml	Gill cpm/mg	Mantle cpm/mg	Hp cpm/mg	Kidney cpm/mg	Gl cpm/mg
A. anatina	2302	307.1 (11.2)	37.2 (11.7)	47.2 (8.6)	35.9 (3.2)	22.0 (2.1)
U. tumidus	9622	466.5 (32.9)	132.3 (18.4)	66.9 (2.7)	314.6 (45.0)	

Means of results from 3 samples of each organ and (SEM) are given. The radioactivity in cpm/ml of the hemolymph of the respective individuals is also given. Hemol., hemolymph; Hp, hepatopancreas; Gl, glochidia.

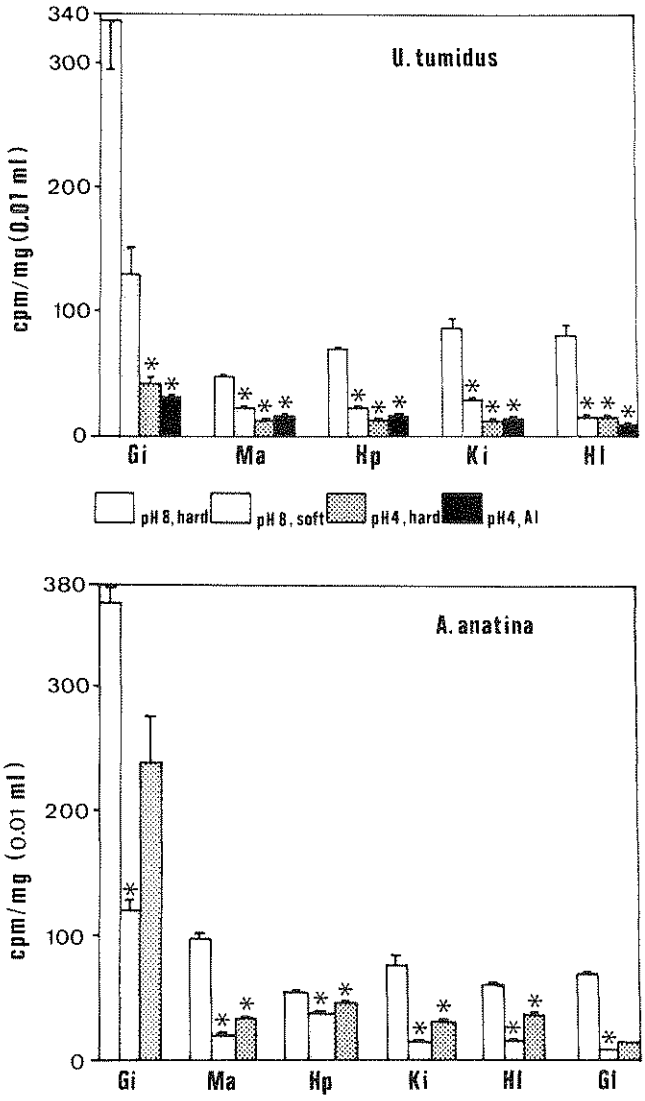

Fig. 1. Accumulation of ^{45}Ca over the course of 24 hrs in the gills (Gi), mantle (Ma), hepatopancreas (Hp), kidney (Ki), and hemolymph (Hl) of *U. tumidus* exposed in circumneutral hard (35 mg Ca/l) and soft (3.5 mg Ca/l) water, and to acid hard (35 mg Cal/l) and acid, aluminum-supplemented (total aluminum 900 μg/l) water. Experimental conditions are described in detail in Table 2. Means ± SEM are given. Asterisks indicate a significant difference between the controls (clams exposed to circumneutral, hard water) and the clams exposed to the other experimental conditions. Accumulation of ^{45}Ca over the course of 24 hrs in the organs (as indicated for *U. tumidus*) and in the glochidia (Gl) of *A. anatina*. Other details as indicated above for *U. tumidus*.

bathing medium (Fig. 1). A positive correlation was found between the concentration of ^{45}Ca in the gills and in the hemolymph (Fig. 2). The ^{45}Ca concentration found in the glochidia of *A. anatina* was about 15% of that in the gills. There was a very significant correlation between the ^{45}Ca con-

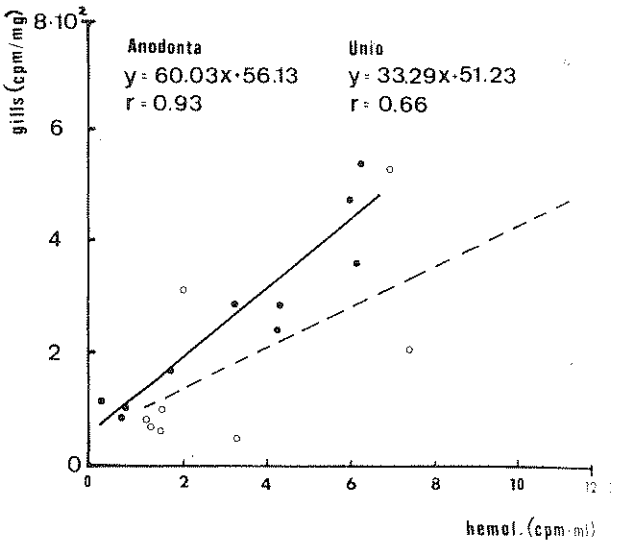

Fig. 2. The linear regression relationships between concentration of ^{45}Ca in the hemolymph and in the gills of *anatina* (filled circles, solid line) and of *U. tumidus* (open cles, dashed line). All data from exposures to circumneu hard and soft water were included.

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centration in the hemolymph and in the glochidia (Fig. 3).

The calcium concretions (CC) in the gills of *A. anatina* accumulated 85% of the total ^{45}Ca four in the gills (172 cpm/mg CC vs. 113 cpm/mg tot gill tissue), and 24% (698 cpm/mg CC vs. 2 cpm/mg gill) in *U. tumidus* (see Table 4). No si nificant differences in uptake of Ca were fou between female and male clams.

Effects of water hardness, pH, and aluminum on uptake of calcium

The rate of uptake of ^{45}Ca by both species unionids was dependent on the ambient ^{45}Ca co centration. A 90% decrease in the ambient ^{45}Ca concentration resulted in an 82% decrease in the amount found in the hemolymph of *A. anatina* after 24 hrs (pH 8, hard vs. pH 8, soft; Fig. 1. the gills the decrease was 68% and in the mant it was 82%. In *U. tumidus* the depletion of from the medium resulted in an 87% decrease accumulation of ^{45}Ca in the hemolymph, a 5 decrease in the gills and a 55% decrease in t mantle (Fig. 1). The decrease in the accumulat of ^{45}Ca was significant in all other organs of *tumidus*, into the exception of the gills (Fig The distribution of ^{45}Ca in the various organs d not differ between clams incubated in hard a soft water.

When compared to the circumneutral cond tions (pH 8), low pH (4.0–4.5) significantly d

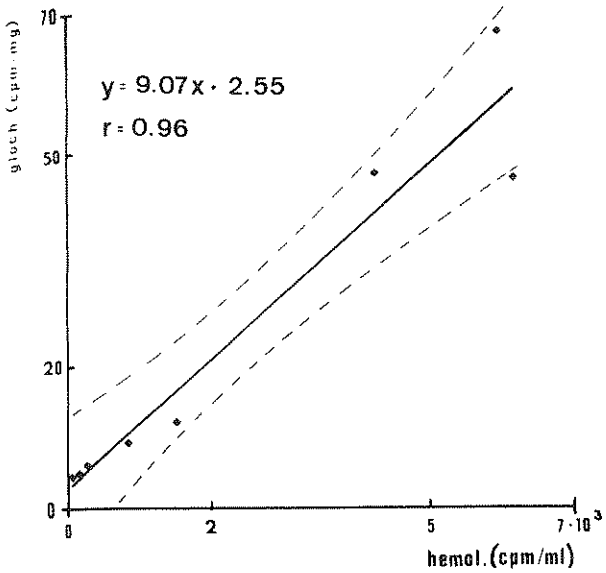

Fig. 3. The linear regression relationship between the concentration of ^{45}Ca in the hemolymph and in the glochidia of *A. anatina*. All data from all exposures were included. The confidence limits are indicated by dashed lines.

creased (by 87%) the amount of ^{45}Ca found in the gills of *U. timidus* (Fig. 1). The decrease in the accumulation of ^{45}Ca in the gills was not significant in *A. anatina* (Fig. 1). In hemolymph the decrease was 61% in *A. anatina* and 80% in *U. timidus*. In the mantle, the decrease was between and 76% in both unionid species. Compared to exposure to acidic conditions alone, the simultaneous exposure of *U. tumidus* to acidic conditions and aluminum did not have any significant, additive inhibitory effect on uptake of calcium (a 90% decrease in the ^{45}Ca concentration of the gills, in the hemolymph and 66% in the mantle). In summary, *A. anatina* appeared more sensitive to salt depletion than to pH, whereas in *U. tumidus* both factors appeared to affect the uptake of ^{45}Ca in the same way.

In the hard, circumneutral medium 13% (*U. tumidus*) to 14.5% (*A. anatina*) of the radioactivity in the bath was taken up by the clams (Table 2). It is difficult to evaluate how much was accumulated in the animal and how much was only adsorbed on the outer shell surface. In the soft medium, 38–45% of the radiolabel in the medium disappeared in 24 hrs. The decreasing concentration gradient between the clam and the ambient medium, caused both by the increase in the ^{45}Ca concentration in the hemolymph and by the disappearance of ^{45}Ca from the bath, may significantly reduce the rate of uptake. In the acid medium, as well as in the acid, aluminum-supplemented medium, the rate of disappearance of the radiolabel from the medium was somewhat reduced, but none of the differences between the groups were statistically significant.

The enrichment of ^{45}Ca in the gills of *A. anatina* and *U. tumidus* was quite similar in hard and in soft water (Table 5), but a difference between the accumulation in the two species was seen in the acid medium. The gills of *A. anatina* could achieve a concentration of 50-fold higher than in the bath, whereas in the gills of *U. tumidus* the concentration achieved was only 10 times higher. In the hemolymph, a ^{45}Ca concentration of at least twice the ambient ^{45}Ca concentration was reached in both circumneutral soft and circumneutral hard medium, but in acid medium the concentration in the hemolymph remained below the ambient ^{45}Ca concentration. Variation in the accumulation of ^{45}Ca among individuals was largest in clams exposed under circumneutral conditions (Fig. 1), while the variation decreased in animals exposed to low pH.

Sources of calcium

Clams were able to gain calcium from their diet as well as from the water (Table 6). The difference in the distribution between organs in the soft parts when clams were exposed to ^{45}Ca-labeled

TABLE 4. Accumulation of ^{45}Ca in the calcium concretions (CC), the total gill tissue and hemolymph of A. anatina *and* U. tumidus *in 24 hrs*

Organ	*A. anatina*	*P*	*U. tumidus*
CC, cpm/mg	172.7 (61.8)	**0.006**	565.4 (83.1)
Gill total, cpm/mg	113.4 (41.4)	0.072	227.6 (32.0)
Hemolymph, cpm/ml	915 (302)	**0.004**	1735 (113)
CC in gills, % w/w	55.8 (3.4)	**0.000**	8.8 (1.2)

The amount of calcium concretions is given as % of organ dry weight.
Means ± SEM of results for 4 clams are given.
P values <0.05 (printed in boldface) indicate significant differences between the species.

TABLE 5. Enrichment of ^{45}Ca in the gills and in the hemolymph (hl) of U. tumidus *and* A. anatina. *Enrichment radioactivity of ^{45}Ca in gills (cpm/mg) or in the hemolymph (cpm/ml)/radioactivity ^{45}Ca in medium (cpm/ml)*

	U. tumidus		*A. anatina*	
	gills	hl	gills	hl
pH 8, hard	73	2.0	90	1.3
pH 8, soft	294	3.4	310	2.4
pH 4–4.5, hard	10	0.4	49	0.6
pH 4–4.5, Al	8	0.6		

When the enrichment for hemolymph was <1, the amount of ^{45}Ca in the hemolymph did not reach the ambient concentration of ^{45}Ca in the course of the 24-hr exposure.

algae or to ^{45}Ca-labeled medium points to different routes of uptake. In both cases, however, gills contained the highest amounts of ^{45}Ca (Table 6), but the other organs, hepatopancreas and mantle accumulated relatively more ^{45}Ca when algae were introduced as a source of ^{45}Ca. The ^{45}Ca concentration in the mantle was almost the same as that in the gills when clams were supplied with labeled algae, whereas when clams were exposed to the radioactive medium, the ^{45}Ca concentration was four-fold higher in the gills than in the mantle.

When the clams were exposed simultaneously to waterborne and dietary ^{45}Ca, the concentrations of Ca in the mantle and hepatopancreas were, when compared to that in the gills, relatively higher than when only waterborne ^{45}Ca was supplied. In the presence of algae, the amount of ^{45}Ca accumulated in the hepatopancreas was significantly higher than that accumulated by clams in the radiolabeled ambient medium. The somewhat higher accumulation in the other organs was not, however, significant.

DISCUSSION

Uptake of calcium in soft parts

Irrespective of the source of calcium, the highest concentrations of ^{45}Ca were always found in the gills. In preliminary studies labial palps were also found to concentrate relatively high levels of ^{45}Ca. The uptake is favored by the fact that both organs are in direct contact with the water pumped through the clam and, as a result of their morphology, they have a large surface area. It is concluded that if the shells are excluded, gills are the main sites of accumulation of Ca, because high amounts (up to 85% in *A. anatina*) of Ca were found in the calcified concretions of the gills.

In *A. anatina* the gills make up approximately 18% of the total soft part of the animal, whereas in *U. tumidus* the gills make up only approximately 10% of the soft part (in terms of dry weight). If gills are the major site of uptake of calcium, then the difference in gill size may have a marked effect on the rate of accumulation of calcium. Since the clams used for the experiments were homogeneous in size, any influence due to the size of individual clams was eliminated.

The glochidial larvae in the water channels of the inner gill demibranches are morphologically isolated from the circulating water (Silverman et al., '87). Therefore, they are able to obtain only a small proportion (8%) of their calcium from the ambient medium. The majority of the calcium needed for the glochidial shells originates from the calcium concretions of the maternal gills (Silverman et al., '87). In the present study, a very significant correlation was found between the Ca concentration in the glochidia and that in the hemolymph. Glochidia found in samples of *A. anatina* were all ripe since, after their removal from the gills, they were able to move their valves

TABLE 6. Accumulation of ^{45}Ca from different sources (dietary, ambient, combination of both) in A. anatina

Source of Ca: Radioactivity of ^{45}Ca in the medium:	Algae (11 cpm/ml)	P_{aw}	Water (3815 cpm/ml)	P_{wwa}	Water + algae (3114 cpm/ml)
Hemolymph	232 (11.4)	**0.001**	5019 (453)	0.159	3807 (60)
Gills	30.5 (3.7)	**0.001**	355.4 (43.4)	0.199	489.9 (95.4)
Mantle	25.2 (6.8)	0.173	92.9 (15.4)	0.106	203.3 (71.6)
Hepatopancreas	15.6 (2.6)	**0.004**	40.8 (7.4)	**0.001**	87.9 (12.6)
Kidney	11.3 (2.8)	0.152	70.8 (16.8)	0.681	62.7 (12.9)

Mean ± SEM of results for 4 clams are given as cpm/ml dry organ (cpm/ml for hemolymph). P_{aw} indicates significant differences between exposures to algae and to water exposures, and P_{wwa} between exposure to water and to water + algae. Significant P values (<0.05) are printed in boldface.

wever, since significant accumulation of ^{45}Ca s found in the glochidia, calcification of the chidial shell obviously continued during the posure period.

These two species of unionids were expected to hibit different affinities for Ca, since according Agrell ('49), *A. anatina* is present in greater ndance in eutrophic water courses, whereas *tumidus* decreases in abundance in eutrophic ter. The calcification of the respective shells lows the same pattern: the shells of *A. anatina* e thickest in the eutrophic waters whereas *U. nidus* forms heaviest shells in oligotrophic wa-s. A less efficient mechanism for uptake of cal-m in *A. anatina* could be a natural explanation this phenomenon. In this study, no difference s found in the efficiency of the uptake of cal-m between these species in the moderately rd water that contained 35 mg Ca/l. Only the a concentration in the hemolymph, both in rd and in soft medium, was somewhat higher in *tumidus*, but the difference was not significant. The correlation between the Ca concentration the hemolymph and in the gills was more sig-icant in *A. anatina*, perhaps because of the dif-rence in the composition of the gills. Only 9% of e dry weight of the gills of *U. tumidus* consists calcium concretions, whereas in the gills of *A. atina* this value is 56%. However, this relative fference is not always found, since the brooding males lose a significant amount of their gill con-etions (Silverman et al., '85). Furthermore, the ct that specimens of both unionid species of nionids were collected from the same area and re, thus, acclimated to the same chemical com-sition with respect to the ambient medium may plain the similarity between the species in rms of the Ca uptake. If the individuals origi-ted from waters with different degrees of water rdness, differences might be found in the effi-ncy of uptake of calcium.

In *U. tumidus*, 22% of the total ^{45}Ca found in e gills was sequestered in the calcium concre-ns, whereas the relative weight of the concre-ns in the gills was only 8.8% of the dry weight the gills. In *A. anatina* the proportion of the a found in the gills was 85% and the relative ight 55.8%, respectively. In both cases the cal-m concretions showed higher affinity for Ca an the rest of the gill tissue. Because of the naller site for sequestration of Ca in the gills of *tumidus*, more Ca may be available for other serves of $CaCO_3$. This possibility might explain part why the shells of the *Unio* species, in particular in waters with low trophic conditions, are heavier than the shells of *Anodonta* species. The higher ^{45}Ca concentration measured in the hemolymph (Table 4) of *U. tumidus* may also be a result of the lower capacity for accumulation (smaller amount of calcium concretions) in the gills. A larger site for sequestration of Ca in the gills of *Anodonta* species may be necessary to fulfill the demands for calcium of the large number of glochidia produced during each reproductive season.

Effect of water hardness, pH, and aluminum

Clams exposed for periods of time to medium of extremely low pH often close their valves in order to avoid contact with the medium (Doherty et al., '87). The decreased uptake of calcium measured in the acid medium of pH 4–4.5 is obviously a result of the valve closure since previous studies have shown (Pynnönen et al., submitted) that low pH significantly affects the normal rhythmic patterns of activity of the adductors. It is possible that, in acid medium, the efflux of Ca ions exceeds the influx of Ca ions, resulting in a net loss of ions. Such loss has been demonstrated in acid-exposed rainbow trout (Reid and McDonald, '88). Since unionid clams are able to withstand hypoxia for at least 6 days (Holwerda and Veenhof, '84), it is obviously profitable for clams under short-term acid stress to close their valves and change over to an anoxic form of metabolism.

A transitory increase in the Ca concentration in the hemolymph, beginning on the second or the third day of exposure to acidic conditions, is found in unionid clams (Pynnönen, '91). This excess of Ca is not derived from the calcium concretions of the gills (Pynnönen, '90), and results from this recent study also rule out the ambient medium as a source of Ca. The only possible source seems to be the shell-mantle pool of calcium. Supporting evidence for this possibility has been provided by Machado et al. ('88), who demonstrated the partial disappearance of the calcium microspherules in the mantle during acute exposure to acidic conditions. Experiments of sufficient duration to show significant decalcification of the shells as a result of an ambient acid stress have not yet been performed, but purely chemical damage to shells leading to microbial contamination has been suggested as a cause of death in *Corbicula fluminea* in natural acid water (Kat 1972). By contrast, Machado et al. ('88) reported shell thickening in *A. cygnea* during a short period of acute pH stress.

In the present study it was demonstrated that, over a 24-hr period, clams under circumneutral conditions were able to take up ^{45}Ca to give concentrations in the hemolymph which were 1.5- (hard water) to 3.5- (soft water) fold higher than the concentrations measured in the ambient medium. When high levels of protons were present in the medium, the ^{45}Ca concentration in the hemolymph after 24 hrs remained below the ^{45}Ca concentration in the medium.

One result of shell closure in bivalves is that $CaCO_3$ is mobilized from the shells in order to buffer the hemolymph during metabolic/respiratory acidosis. Simultaneously, deposition of calcium in the shells is inhibited (Crenshaw and Neff, '68). Both factors together lead to an increased Ca concentration in the hemolymph. In soft water, the concentration gradient between the medium and hemolymph was relatively steep (hemolymph contained approximately 50- to 70-fold more Ca than the medium). Under these circumstances an additional acid stress, leading to an increase in the Ca concentration in the hemolymph, may create a concentration gradient that totally inhibits uptake of calcium from the medium.

Factors such as cadmium and manganese have been reported to inhibit uptake of Ca in fish and crustaceans (Reader and Morris, '88, Wright, '80). Low pH depresses the uptake of Ca in salmonid fish (Reader and Morris, '88) as well as in crustaceans (Malley, '80), while a simultaneous or separate exposure to aluminum does not affect Ca influx in clams (Malley and Chang, '85). A relatively high total concentration of aluminum (900 μg/l) had only a minor, additional inhibitory effect on the uptake of Ca by *U. tumidus*. However, at pH 4 almost all the aluminum is in the free cationic form (Al^{3+}); only 230 parts per billion of aluminum (at 25°C) can be totally solubilized in water (McDonald et al., '89). Thus, the effective (dissolved) concentration of aluminum was obviously much lower than 900 ppb. As a result of the change in the pH during the exposure, changes could occur in the partitioning of the aluminum. It is possible that the toxic effects of aluminum could be studied more easily in a medium with a higher pH since, in crustaceans, more than 90% of the uptake of calcium below pH 5.0 is inhibited by the acidic conditions alone (Malley, '80).

It is highly probable that inhibition of uptake of Ca that resulted from exposure to low pH was caused primarily by shell closure. Even in the soft medium, shell closure could affect the rate of uptake of Ca to some extent, since clams may avoid contact with a dilute medium in order to diminish the loss of electrolytes. Other factors, in addition to shell closure, may lie behind the depressed uptake of Ca (Malley, '80). High external concentration of protons may interfere with the exchange of internal protons for external Ca ions, or it may directly affect the active transport system of the membrane. In addition, at pH 4–4.5, too few HCO_3^- ions are available to accompany incoming Ca ions for the maintenance of electrical neutrality.

Sources of calcium

In this study it has been shown that *A. anatina* is able to obtain Ca from water as well as from the diet. The fact that the hepatopancreas accumulated relatively more ^{45}Ca than the gills when dietary ^{45}Ca was available proved that uptake of ^{45}Ca took place through the gut. Aquatic gastropods are also able to fulfill their calcium requirements using both waterborne and dietary calcium (van der Borght and Puymbroeck, '66). The ability of freshwater molluscs to utilize dietary Ca can be of great importance in the poorly buffered soft-water areas of Scandinavia and North America, where the concentration of available waterborne calcium is less than 1 mg/l.

The clams exposed simultaneously to waterborne and dietary ^{45}Ca accumulated more ^{45}Ca than did clams exposed to waterborne ^{45}Ca only even though the total radioactivity of the bath was lower. There may be a simple explanation for this phenomenon. The presence of algae in the medium stimulates filtration by the clams (Morton, '83). This stimulation may result in the increased uptake of ^{45}Ca. In addition to food particles, factors such as temperature (DeBruin & Davids, '70), diurnal rhythm (Salánki, '64), tidal cycle (Morton, '70) and chemical factors (Doherty et al., '87) may alter the rate of filtration and thereby, affect the rate of uptake of calcium.

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