Ann. Zool. Fennici 32: 355–363
Helsinki 1 November 1995
ISSN 0003-455X

Changes in acid-base status, gases and electrolytes in the hemolymph of freshwater unionids during continuous and intermittent exposure to acid water

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Received 17 July 1994, accepted 1 April 1995

The effect of low ambient pH (pH 4.0–4.5) on the levels of hemolymph gases (pCO_2, pO_2), the acid-base status and electrolytes in the hemolymph was investigated in two freshwater unionids (*Anodonta anatina* and *Unio tumidus*) under moderately hard water (17 mgCa/l) conditions. In order to simulate freshwater acidification during heavy rainfall or spring snow-melt, a continuous 15-day exposure and an intermittent exposure regime (6 hr exposure, 18 hr recovery continued for 48 hr) were introduced. During the 15-day low pH exposure, an initial reaction of both clam species to acid stress was a decrease in blood pO_2 concentration after only 3 hr of exposure. At the same time the pH of the hemolymph decreased rapidly. In *U. tumidus*, the compensation by means of $CaCO_3$ release restored the original hemolymph pH level in 3 days, whereas in *A. anatina*, full compensation for the acid-base disturbance took more than 8 days. Continuous acid exposure decreased the hemolymph Na^+ and Cl^- levels in both species, the decrease being more severe in *U. tumidus*. Intermittent pulses of acid water affected the hemolymph gases and acid-base status, but not the concentration of electrolytes.

1. Introduction

Due to anthropogenic acid emissions, freshwater organisms living in waters of poor buffering capacity are often exposed to acidic conditions. Most of the species are severely affected when the water pH falls below 5.0 (Økland and Økland 1986). In previous studies on acid stress in freshwater bivalves, it was demonstrated that unionids are able to protect themselves against low ambient pH by closing their valves and temporarily changing over to anaerobic metabolism (Pynnönen and Huebner, unpublished). The survival of *A. anatina* under cold, anoxic conditions can extend to 67 days (Englund and Heino 1992).

As a reaction to acid exposure, the hemolymph of unionids shows a transitory elevation of Ca^{2+}, whereas the concentrations of the other blood ions decline (Pynnönen 1990a). The trigger for this increase in Ca^{2+} concentration is obviously the decrease in hemolymph pH. As a result of addition of acid and aluminum to a lake, Malley

et al. (1988) have demonstrated a simultaneous increase in the circulating Ca^{2+} and HCO_3^- in the blood of *Anodonta*, which strongly suggests the use of $CaCO_3$ reserves to buffer the hemolymph. The internal origin of the $CaCO_3$ used to compensate metabolic acidosis is supported by the fact that the uptake of Ca from the ambient medium is severely impaired in acid water (Pynnönen 1991a). The acidification of hemolymph is probably of both respiratory and metabolic origin, since unionids, when exposed to the low water pH (> 5.0) tend to close their valves (Pynnönen 1991), which due to accumulation of CO_2 leads to respiratory acidosis.

Buffer systems other than the CO_2^- bicarbonate are obviously of minor importance in freshwater unionids, because these molluscs lack respiratory pigments and possess a hemolymph which is very dilute in both mineral solutes and amino acids (Potts 1954). A requirement for an effective CO_2^- bicarbonate buffer system is that the pCO_2 in body fluids can be regulated via a gas exchange mechanism (Heisler 1989). Changes in pCO_2 may be regulated by ventilation to compensate for non-respiratory acid-base disturbances (Heisler 1989), but the demand for oxygen often determines the rate of ventilation in many aquatic animals and restricts their capacity to regulate the amount of CO_2 in blood and thus decreases the effectiveness of the open bicarbonate buffer system. Epithelial contact with the surrounding water, however, facilitates the ion transfer mechanisms, which may serve to support the acid-base regulation.

In this study, the effects of low pH stress (pH 4.0–4.5) on acid-base regulation, electrolyte balance and hemolymph pO_2 in freshwater bivalves were studied during a 15-day exposure under relatively hard water conditions (17 mg Ca/l). Since in earlier studies the changes in hemolymph pH and electrolyte concentrations under identical low pH conditions (Pynnönen 1990a) were different *Anodonta* and *Unio* species, differences in the regulation of acid-base balance under a severe acid stress could be expected.

A continuous 15-day exposure was conducted in the present study in order to simulate chronic exposure to low water pH. In nature, a chronic acid exposure could be caused by an extended period of heavy rainfalls such as those occurring in the fall. During the daytime in the spring, acid meltwater temporarily decrease the pH of the watershed, whereas at night circumneutral conditions predominate. An intermittent low pH exposure was used to mimic this kind of fluctuating pH conditions during the period of snowmelt.

Table 1. Properties and composition of the laboratory tapwater.

Parameter	
colour, Pt mg/l	0
conductivity, mS/m	13.9
pH, range	7.8–8.0
temperature, °C	9.0
alkalinity, mmol/l	0.64
bicarbonate, mg/l (mmol/l)	38.8 (0.63)
hardness, °dH	3.0
nitrate, NO_3^-, mg/l (mol/l)	0.28 (0.0004)
sulphate, SO_4^{2-}, mg/l (mmol/l)	7.5 (0.07)
chloride, mg/l (mmol/l)	6.4 (0.18)
calcium, mg/l (mmol/l)	17.1 (0.42)
magnesium, mg/l (mmol/l)	1.6 (0.07)
potassium, mg/l (mmol/l)	1.4 (0.03)
sodium, mg/l (mmol/l)	5.2 (0.22)
aluminium, mg/l (mmol/l)	0.06 (0.003)

2. Material and methods

Freshwater clams, *Anodonta anatina* L. and *Unio tumidus* L. were collected in June 1992 from one location from the Vantaa River, in a grassland watershed situated in southern Finland. At the time of collection, the water temperature varied between +18°C and +20°C. The water Ca concentration at this time was 28 mg/l.

Before the exposures, the clams were kept for one week in an aquarium without substratum, with continuously-flowing filtered tapwater (+9°C, pH 7.8). The chemical composition of tapwater is given in Table 1. During the acclimation period, the clams were not fed. Specimens with shell lengths of 60 to 90 mm (*A. anatina*) and 61 to 94 cm (*U. tumidus*) were used.

2.1. Continuous acid exposure

The clams were exposed for 15 days in two identical stainless steel aquaria each containing a static volume of 150 l of moderately hard (17 mg Ca/l, 3.0°H) water for 15

days. Continuous aeration was maintained and 90% of the entire water volume was changed every third day. In the first aquarium (experimental), the water pH was adjusted to between 4.0 and 4.5 using 1 M sulfuric acid and the pH was checked daily using a KCl electrode. In the second aquarium (control) the ambient pH varied between 7.2 and 7.4. Control and experimental tests were run simultaneously under identical temperature (+9°C) and light (natural light schedule) conditions. The parameters for the exposure water are given in Table 1. One hundred clams (50 *A. anatina* and 50 *U.tumidus*) were exposed in both tanks. No deaths occurred during the exposures.

Prior to the onset of exposure, on day zero, samples were taken from six animals of both species. After 3, 6, and 24 hours and 3, 8 and 15 days of acidic exposure, six clams of each species from both tanks (control and experimental) were taken for analysis. The hemolymph samples were taken from the sinus of the anterior adductor muscle using a 1 ml syringe fitted with a 22 gauge needle. The total sample volume taken from one individual was 1 ml. The pH, total CO_2 ($totCO_2$, mmol/l) and the partial pressure of oxygen (pO_2, mmHg) of the hemolymph were measured immediately after sampling. The remainder of the sample was frozen and kept at –20°C for later electrolyte analysis.

The pH and the $totCO_2$ concentration of the hemolymph were measured using a Radiometer BMS3 Mk2 PHM73 pH/blood gas monitor thermostated at the experimental temperature (+9°C). The $totCO_2$ of the hemolymph was measured using the method of Cameron (1971). The HCO_3^- concentration of the hemolymph was calculated from the pH and the $totCO_2$ concentration using the Henderson-Hasselbalch equation (pKa = 6.39). The pCO_2 was calculated using the equation $CO_2 = \alpha \times pCO_2$, where α = 0.0671542. The pKa and α values were according to Massabuau et al (1984). The oxygen tension (pO_2) of the hemolymph was measured using a Radiometer BMS3 Mk2 PHM72 system at 9°C.

The Na^+ and K^+ concentrations in the hemolymph were determined simultaneously in diluted samples byß an EEL flame photometer and the Cl^- concentrations from undiluted samples using a Radiometer CMT 10 chloride titrator. Ca^{2+} and Mg^{2+} concentrations in the hemolymph were measured using colorimetric methods for calcium and magnesium (WAKO test kit B 997-21809 for calcium and test kit B 999-83909 for magnesium).

The differences in hemolymph gases, pH and ionic composition between experimental and control animals were tested for significance using the two-way Student's *t*-test. A probability limit of $P \leq 0.05$ was considered as significant.

2.2. Intermittent acid exposure

Thirty *A. anatina* and 30 *U. tumidus* were exposed intermittently to low pH in moderately hard water containing 17 mg Ca/l. A low pH pulse of 6 hr duration was repeated twice, starting at 09.00. The low pH of 4.0–4.5 was reached gradually, over approximately 30 minutes, by adding carefully small amounts of sulphuric acid to the experimental tank. The continuous aeration mixed the acid in the entire water volume, thus no extra stirring was necessary. During the low pH period of 6 hr, the animals were kept in static conditions and continuous aeration was maintained. During the 6-hr period, the pH remained static without readjustment. Following the low pH period (at 15.00), the ambient pH of the aquarium was allowed to increase to the circumneutral pH level (pH 7.2–7.4) by reconnecting the water flow. A recovery period of 18 hr under circumneutral pH conditions followed the low pH period. The regime was repeated the following day.

Prior to the onset of the first low pH pulse, 6 clams of both species were sampled for zero time controls. After both 6-hr acid exposures and 18 hr recoveries, 4 clams from both species were sampled. The pH, pCO_2, pO_2, Ca^{2+} and HCO_3^- concentrations in the hemolymph were measured and calculated as described above.

The differences in hemolymph gases, pH and ionic composition between experimentals and controls (zero time points) were tested for significance using the two-way Student's *t*-test.

3. Results

3.1. Continuous 15-day acid exposure

3.1.1. Hemolymph acid-base status

The pH of the hemolymph decreased significantly after 6 hr of exposure and remained at this new lower level during the next 8 days in *A. anatina* but only during the next 24 hr in *U. tumidus* (Fig. 1). The initial pH decrease during the first 6 hr of exposure was about the same order of magnitude (approx. 0.4 pH units) in both species. The lowest individual pH values measured were: pH 7.69 in *U. tumidus* after 24 hr of exposure and pH 7.57 in *A. anatina* after 72 hr of exposure.

During the first 3 hr of acid exposure, the hemolymph pH of *A. anatina* decreased without any changes in the hemolymph [HCO_3^-] and pCO_2 levels (Fig. 2). After 6 hr of exposure, the compensation by $CaCO_3$ increased hemolymph [HCO_3^-] and pCO_2 without affecting the hemolymph pH. This temporary steady-state, where pH was maintained between 7.5 and 7.7, was sustained for the next 7 days. The pH of the hemolymph reached the

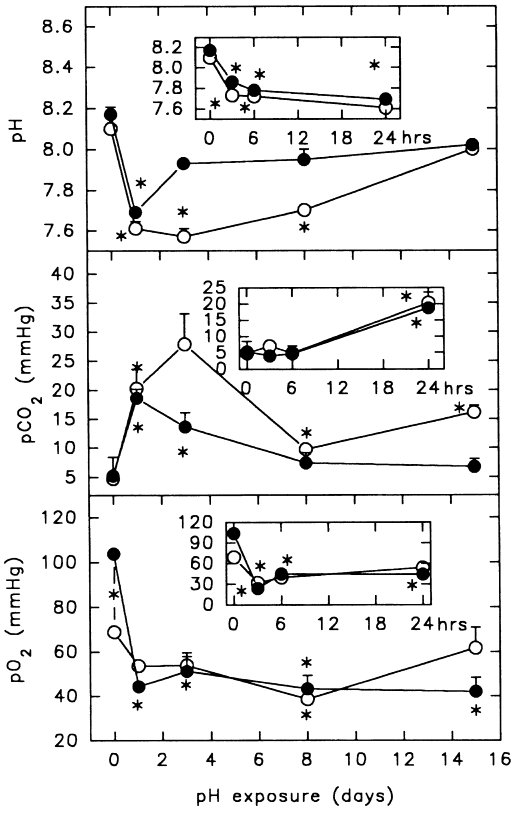

Fig. 1. Changes in pH, carbon dioxide (pCO2) and oxygen (pO_2) partial pressures in the hemolymph of *A. anatina* (open circles) and *U. tumidus* (closed circles) during a 15-day acid exposure (pH 4.0–4.5). Insert figures show changes during the first 24 hr of acid exposure. Asterisks indicate values significantly different ($P \leq 0.05$) from the controls kept under circumneutral conditions. Data are means ±*SEM*; for controls and 24, 48, and 72 hr exposed $N = 6$, for others $N = 4$.

initial value between 8 and 15 days from the onset of acid exposure (Fig. 1).

In *U. tumidus*, the initial shift in acid-base status during the first 6 hr of exposure took place at constant pCO_2 (6 mmHg), thus only the pH and $[HCO_3^-]$ were decreased (Fig. 3). The pattern of buffering of the hemolymph of *U. tumidus* by $CaCO_3$ release was very similar to the one seen in *A. anatina*: hemolymph $[HCO_3^-]$ increased by 20 mmol/l during the next 18 hr (Fig. 3). The pH of the hemolymph returned to the control level after 3 days of exposure (Fig.1). The $[HCO_3^-]$ and the pCO_2 level of the hemolymph were normal after 8 days of acid exposure (Fig. 1 and Fig. 4).

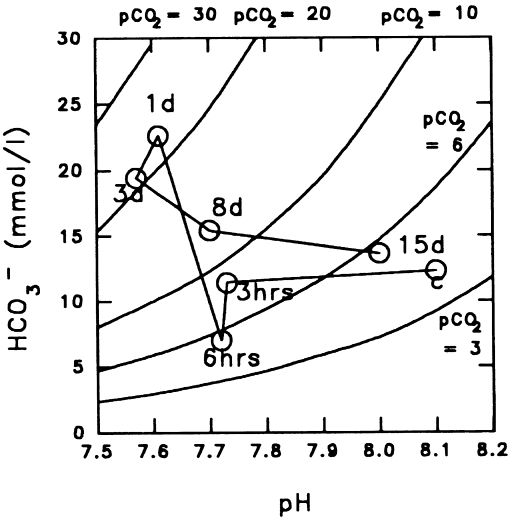

Fig. 2. Hemolymph $[HCO_3^-]$ vs pH diagram showing the changes in extracellular acid-base status in *A. anatina* during 15 day exposure to pH 4.0–4.5.°C = controls at day 0. The pCO_2 isopleths are in mmHg.

3.1.2. Hemolymph oxygen tension

The pO_2 in the hemolymph differed significantly between the two unionid species under normoxic, circumneutral conditions: *U. tumidus* had pO_2 value of 104 mmHg and *A. anatina* a value of 69 mmHg (Fig. 1). During the acid exposure, the pO_2 in both species decreased significantly. In *U. tumidus*, the lowest pO_2 value measured during the acid stress was 24 mmHg and in *A. anatina*, 32 mmHg. Both of these minimum values were measured after 3 hr after the onset of acid exposure. In *U. tumidus*, pO_2 of the hemolymph remained throughout the exposure at a significantly lower level than the control values, whereas in *A. anatina*, significantly lower pO_2 values were measured after 3 hr and 8 days from the onset of exposure. At the end of the 15-day exposure, the pO_2 of *A. anatina* was again at the control level (Fig. 1).

3.1.3. Hemolymph electrolyte concentrations

24 hr after the onset of acid exposure, the Ca^{2+} concentrations of the hemolymph of both species were significantly higher than the control values (Fig. 4). Between 1 and 15 days of exposure, the hemolymph HCO_3^- concentration correlated positively with the Ca^{2+} concentration in *A. anatina*

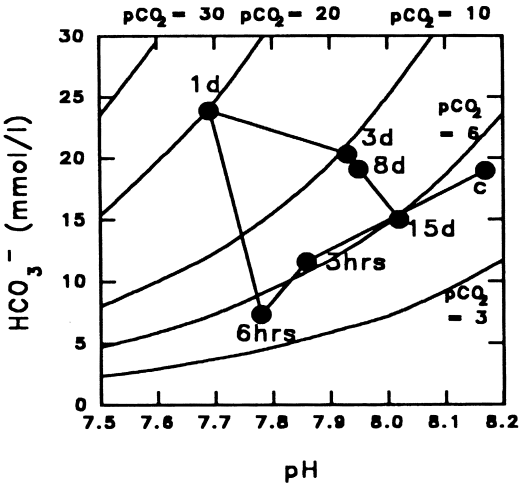

Fig. 3. Hemolymph [HCO_3^-] vs pH diagram showing the changes in extracellular acid-base status in *U. tumidus* during 15 day exposure to pH 4.0–4.5.°C = controls at day 0. The pCO_2 isopleths are in mmHg.

but not in *U. tumidus* (Fig. 5). During the first 24 hr of exposure, the HCO_3^- concentration in the hemolymph decreased significantly in both species. Prior to the exposures, the hemolymph Ca^{2+} concentration did not differ significantly between the two species, whereas the hemolymph HCO_3^- concentration was significantly higher in *U. tumidus* than in *A. anatina* (Fig. 4).

A significant decrease in the hemolymph Na^+ concentration was measured in *U. tumidus* 1 and 3 days after the onset of acid exposure (Fig. 6). In *A. anatina*, the hemolymph Na^+ values were not affected. As with HCO_3^-, a significant difference between the species was noted in the hemolymph Na^+ and K^+ concentrations (Fig. 6). Under circumneutral conditions, the concentrations of both ions were twice as high in *U. tumidus* as in *A. anatina*. After 24 hr of exposure, a significant, but transient, increase in K^+ concentration of the hemolymph was seen in *A. anatina*, whereas the hemolymph K^+ concentrations in *U. tumidus* did not change during the acid exposure (Fig. 6).

3.2. Intermittent acid exposure

Low pH peaks of 6 hr decreased significantly the pH level of the hemolymph in *A. anatina* as also in *U. tumidus* (Fig.7). During the circumneutral 18 hr recovery periods, the hemolymph pH re-

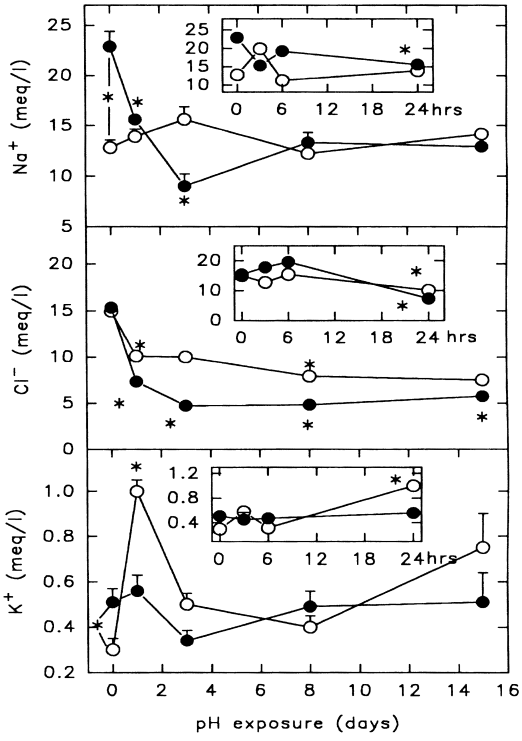

Fig. 4. Changes in the Ca^{2+}, HCO_3^- and Mg^{2+} concentrations in the hemolymph of *A. anatina* (open circles) and *U. tumidus* (closed circles) during a 15-day acid exposure (pH 4.0–4.5). Insert figures show changes during the first 24 hr of acid exposure. Other details as in legend to Fig. 1.

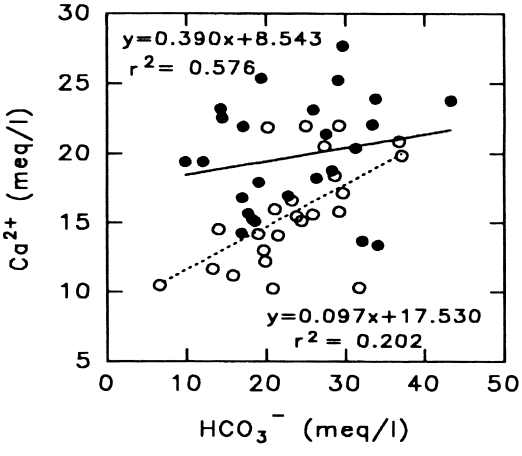

Fig. 5. The linear regression relationships between the Ca^{2+} and HCO_3^- concentrations in the hemolymph of *A.anatina* (open circles, dotted line, $r^2 = 0.576$) and *U. tumidus* (closed circles, solid line, $r^2 = 0.202$) based on the data from individuals during acid exposure (days 1, 3, 8 and 15).

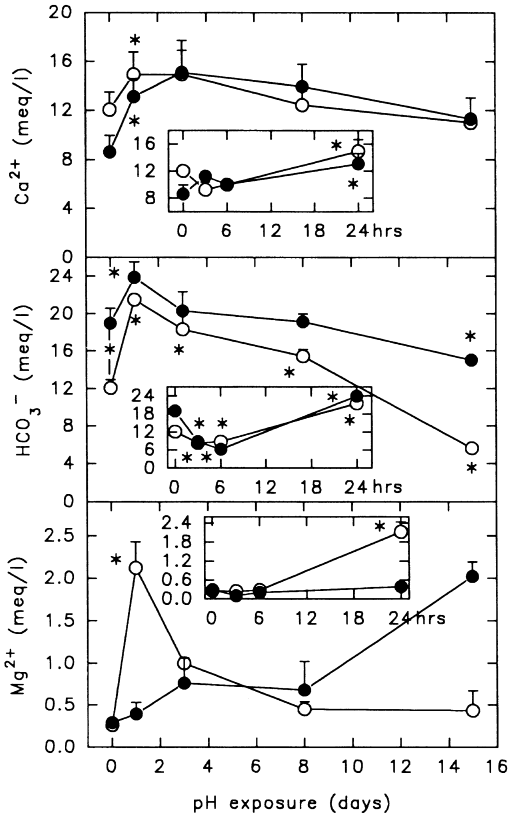

Fig. 6. Changes in the Na^+, Cl^- and K^+ concentration in the hemolymph of *A. anatina* (open circles) and *U. tumidus* (closed circles) during a 15-day acid exposure (pH 4.0–4.5). Inserted figures show changes during the first 24 hr of acid exposure. Other details as in legend to Fig. 1.

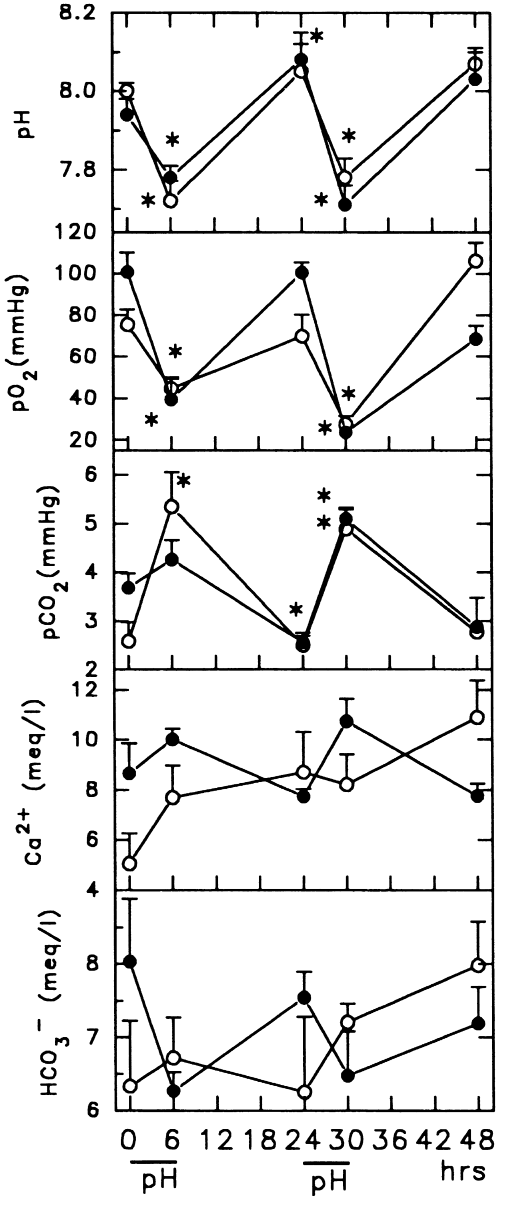

Fig. 7. Changes in the pH), oxygen (pO_2) and carbon dioxide (pCO_2) partial pressure and Ca^{2+} and HCO_3^- concentration in the hemolymph of *A. anatina* (open circles) and *U. tumidus* (closed circles) during intermittent acid exposure (6 hr pH 4.0–4.5: 18 hr circumneutral pH). Solid line indicates the duration of acid exposure, dotted line the circumneutral recovery period. Asterisks indicate values significantly different ($P \leq 0.05$) from the controls sampled before the onset of exposure. Data are means ± *SEM*; $N = 4$.

covered to its normal level. During the first recovery period, the pH of the hemolymph in *U. tumidus* rose even slightly above the control level (Fig.7).

In both unionid species, hemolymph pO_2 fell below the control level during the low pH period. Similarly, during the recovery period, normal hemolymph pO_2 values were restored in *A. anatina* as well as in *U. tumidus*. A negative correlation between the hemolymph pH and pCO_2 was found: when the pH decreased significantly, the blood pCO_2 concentrations rose significantly. This was, however, the case only in *A. anatina*. In *U. tumidus*, the first acid period did not affect the hemolymph pCO_2 level, but the second acid period significantly increased the pCO_2 of the hemolymph. Following the first recovery period, a significant increase in blood pH occurred simultaneously with a significant decrease in hemolymph pCO_2.

During the intermittent exposure, electrolytes other than Ca^{2+} and HCO_3^- were not determined, because in the earlier studies (Pynnönen 1991b) it was seen that short repeated exposures (up to 3 days) to acid water did not affect Na^+ and K^+ concentrations in unionid hemolymph. Small increases of hemolymph [HCO_3^-] and [Ca^{2+}] were measured in this study (Fig. 7), but the levels of these ions were not significantly affected.

4. Discussion

During continuous 15-day exposure, the pH of the hemolymph of both species decreased significantly already after 3 hrs of exposure. This was accompanied by a decrease in the hemolymph pO_2. An increased diffusion distance across gill epithelium could explain the decrease in the hemolymph oxygen content. A mucus on the gill surface or hypertrophication of the gill epithelium could lie behind this phenomenon. On the other hand, in *Anodonta cygnea*, the time spend with valves open decreases significantly when the water pH falls below 5.0 (Pynnönen 1991c). In recent studies we also found pH 4.3 to decrease the time *A. anatina* spends with valves open by 60% (Pynnönen and Englund, unpublished results).

After 6 hr of acid exposure, dissolved $CaCO_3$ material buffered the excess of the H^+ ions and elevated the [HCO_3^-] of the hemolymph. This significantly elevated the hemolymph pCO_2. In *U. tumidus*, the compensation by means of HCO_3^- buffering re-adjusted the pH of the hemolymph to the control level in 24 hr, whereas, in *A. anatina*, it took more than 8 days to reach the pH level of the control animals.

The pH values measured, before the hemolymph HCO_3^- concentration started to rise, were 7.57 in *A. anatina* and 7.69 in *U. tumidus*, which suggest that the pH level which causes the dissolution of $CaCO_3$ lies between pH 7.5 and 7.7. Heisler (1986) suggests that there is a 30 mmol/l upper limit for [HCO_3^-], which is determined either by the sensitivity of kidney or gills. In this study the highest HCO_3^- concentrations measured were 23.4 mmol/l in *A. anatina* and 27.7 mmol/l in *U. tumidus*. These values were measured after 24 hr of exposure in *U. tumidus* and after 72 hr of exposure in *A. anatina*. The HCO_3^- concentration in the body fluids of aquatic animals normally accounts for between 3 and 15% of the total anions (Cameron and Iwama 1989). In unionids, HCO_3^- represents 40% of the total anions in *A. anatina* and 55% in *U. tumidus*, and, in addition, both species possess large buffer stores in their soft parts and shells. Thus it is questionable if the difference between the amount of the buffer material could explain the greater capacity of *U. tumidus* to regulate the extracellular acid-base balance.

In fish, the compensation for hypercapnia-induced acid-base disturbance is much more rapid in water with a higher ionic content (Janssen and Randall 1975, Eddy et al 1977). In *U. tumidus*, the acid-base disturbance caused by acid stress was corrected within 3 days of exposure to moderately hard water (this study), whereas, under soft-water-conditions, no compensation was detected during 8 days of acid exposure (Pynnönen, 1994). In *A. anatina*, no compensation of the hemolymph pH was achieved either during 8 days in acid, soft water or during 15 days in acid, moderately hard water. The soft water exposure alone changed the ionic composition of the hemolymph, but it did not affect the acid-base balance (Pynnönen, 1994).

A stronger correlation between the increase of pO_2 and the decrease of pCO_2 was expected. This was the case only in *A. anatina*, not in *U. tumidus*. In *U. tumidus*, when the pCO_2 decreased (and pH increased), no significant increase in pO_2 was measured. This may be explained by the fact that the solubility of CO_2 of water is 30 times higher than the solubility of O_2, and since the experimental water was through continuous aeration practically decarbonated, only a rapid water circulation through the mantle cavity was needed to get rid of the excessive CO_2. This kind of short-time shell opening may not have been long enough to affect on the pO_2.

The K^+ concentration in the hemolymph of *A. anatina* increased 3-fold after 24 hr of acid exposure. This K^+ could be originating from the intracellular compartment, which lost K^+ to the acidifying extracellular space. The significant increases in hemolymph [Mg^{2+}], measured after 15 days of acid exposure in *U. tumidus*, and after 24 hr of exposure in *A. anatina*, were likely due to shell dissolution.

Due to the lower capacitance coefficient ratio for O_2/CO_2 in water compared to air, both fish and molluscs have to face the same restriction in bicarbonate buffering. However, athough there are some extremely anoxia-tolerant fish species, oxygen is less essential for molluscan metabolism and thus the demand for O_2 does not restrict the operation of the open bicarbonate buffering to the same extent as it does in fish. In addition, molluscs, when compared to fish also possess other physiological advantages. Clams can utilize large $CaCO_3$ reserves and their high anaerobic capacity allows the avoidance of acid stress by means of shell closure. In addition, clams can, by means of shell closure, at least diminish the influx of H^+ ions and the efflux of electrolytes.

A disadvantage for clams is that shell formation demands continuous elimination and buffering of the protons from the extrapallial fluid. This acid load is also removed by bicarbonate buffering (Wilbur and Saleuddin 1983). High metabolic acid load caused by respiratory or acid stress may totally inhibit shell formation. Besides this, restricted mobility of the molluscs diminishes the opportunity to actively avoid local acidification.

A question arises if protective shell closure is more favorable than non-restricted ventilation in case of short-term acid stress. If the clam ventilates in acid medium, H^+ ions can freely enter the clam and increased amounts of $CaCO_3$ must be used in order to buffer the hemolymph. The amounts of Ca^{2+} and HCO_3^- lost to the surrounding medium during continuous ventilation in acid water are not known, but it has been shown (Pynnönen 1991a) that at least the uptake of Ca is inhibited in acid water. Although freshwater unionids have large $CaCO_3$ reserves, a minor decrease in the $CaCO_3$ reserves of individuals living in waters low in Ca could severely affect both reproduction and shell growth of these organisms.

The duration of acid stress is the factor which most likely favours certain behavioral strategies. During an intermittent acid stress, which is common during the time of snowmelt, shell closure during the acid peak is advantageous, since this diminishes the electrolyte loss, if only it does not increase the accumulation of harmful metabolites up to levels which could be detrimental to clams. Since Anodonta at low water temperatures (+4–5°C) is extremely insensitive to accumulation of metabolites (Englund and Heino, 1992) and has a stronger tendency than Unio to keep valves closed (Englund and Heino, 1994), protective shell closure is the most obvious strategy during the spring snow-melt with low water temperatures. In addition, avoidance by means of shell closure also diminishes the accumulation of harmful substances, e.g. heavy metals (Pynnönen 1990b, 1990c), which are often present at high concentrations in the acidified freshwaters.

Acknowledgments. This work was financially supported by The Finnish Academy of Sciences.

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