Changes in haemolymph ion concentrations of *Astacus astacus* L. and *Pacifastacus leniusculus* (Dana) after exposure to low pH and aluminium

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Abstract

During exposure to soft water, acidified to pH 4.0, the haemolymph concentrations of Na^+, K^+, and Cl^- decreased whereas the Ca^{2+} concentration fluctuated in *Astacus astacus.* The haemocyte content of K^+ decreased from 9% to 2% of the total haemolymph K^+ content after exposure to pH 3.7 for 3 days. Within 14 days, 250 $\mu g\ Al^{3+}\ l^{-1}$, as $Al_2(SO_4)_3$ at pH 5.0, reduced the haemolymph Na^+ content in *Astacus astacus* and *Pacifastacus leniusculus,* however, the effects were less pronounced than earlier reported for fish. Disturbed ion regulation, mainly depending on low pH, is thought to contribute to the absence of these species in acid waters.

Introduction

Effects of lake acidification on freshwater organisms have been thoroughly studied in both northern Europe and North America during the last ten years (e.g. Beamish & Harvey, 1972; Leivestad *et al.,* 1976; Schofield, 1976; Almer *et al.,* 1978). Low pH affects ion regulation in most freshwater organisms, as reported for fish by Packer & Dunson (1970), Leivestad *et al.* (1976), McWilliams (1980), McDonald & Wood (1981), Runn (1982) and several others. In decapods, mechanisms of ion regulation have been thoroughly studied (see Mantel & Farmer, 1982, for review), and effects of acid water on ion regulation of crayfish has been reported by Malley (1980) *(Orconectes virilis),* Morgan & McMahon (1982), McMahon & Morgan (1983) *(Orconectes rusticus* and *Procambarus clarkii),* and Appelberg (1984) (early stages of *Astacus astacus*). However, the effect of acidification on decapods is less well understood than it is on fish.

There is strong evidence that aluminium acts as a toxic agent in acidified natural waters, since it causes disturbances of ion regulation and blood oxygen tension in fish at sublethal pH levels (Leivestad *et al.,* 1976; Dickson, 1978; Baker & Schofield, 1980; Muniz & Leivestad, 1980a, b). It is mainly the inorganic aluminium fraction that is thought to be toxic to fish, and the effect is most pronounced in soft water at pH 5.0 to 5.5 (Muniz & Leivestad, 1980b; Dricsoll *et al.,* 1980). Although the drastic effects of aluminium on fish in acid water are well established, effects of aluminium on crayfish has not been reported.

In the present paper, the effect of exposure to acid water on the ion balance between haemolymph and the ambient water and on the ion balance between haemocyte and plasma of *Astacus astacus* has been studied. The effect of exposure to aluminium at pH 5.0 on the haemolymph ion concentration of *Astacus astacus* and *Pacifastacus leniusculus* is also reported.

Material and methods

Three experiments were conducted. In experiment 1, eight *A. astacus* were exposed to water at

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pH 3.7, and sampling of haemocytes and plasma was made before exposure and after three days. In experiment 2, three groups of eight *A. astacus* were exposed to pH 6.8 (control), 5.0, and 4.0 for 11 days, and sampling of haemolymph was performed on days 0, 4, 8, and 11. In the third experiment the effect of aluminium at pH 5.0 was tested on both *A. astacus* and *P. leniusculus* at concentrations of 0, 250, 500, and 1 000 μg Al^{3+} l^{-1} (0, 9.3, 18.5, and 37.0 mmol l^{-1}, respectively) for 14 days, using four individuals of each species in each group.

The *A. astacus* originated from the non-acidified lakes Tomtsjön (experiments 1 and 3) and Tisaren (experiment 2), and the *P. leniusculus* from Lake Erken, likewise non-acidified. All crayfish were caught in the autumn and kept in large plastic tanks at 10 °C, supplied with tap-water, during the winter. Only specimens that were regarded to be in intermoult stage (C4) were used in experiments. The time for acclimation to the experimental water and temperature was 6 days in experiments 1 and 3, and 8 days in experiment 2. The animals were not fed during the time of acclimation, nor during the experimental periods.

In experiment 1 the temperature, during the acclimation and experimental periods, was room temperature (20 °C), and in experiments 2 and 3 it was 16.0 ± 1.0 °C.

To obtain water of constant quality, deionized water supplied with salts was used in all experiments. The major ions, as added, were Ca^{2+} 0.090 mmol l^{-1}, Mg^{2+} 0.030 mmol l^{-1}, Na^{+} 0.090 mmol l^{-1}, K^{+} 0.015 mmol l^{-1}, HCO_3^-, Cl^-, and NO_3^- all 0.090 mmol l^{-1} and SO_4^{2-} 0.045 mmol l^{-1}. The measured conductivity was 4.8 mS m^{-1}, pH 6.8, and the calculated content of pCO_2, after acidification of the water, was less than 2.5 mm Hg. $Al_2(SO_4)_3 \times 18\ H_2O$ (Mallininckrodt Chemical Works) was used to create different concentration of Al^{3+} in the medium.

In experiment 1, a single 80 l aquarium was used. In experiments 2 and 3, an aquarium system composed of four separate units, consisting of four 13 l plastic beakers, connected to an 80 l aquarium, where replacements of water and pH adjustments were made. Two crayfishes were placed in each plastic beaker, and a circulating system between these and the aquarium was complemented with an extra aeration of the water. In experiments 1 and 2 the water was filtered, recirculated, and aerated, and half of the water volume was removed every third day. In experiment 3, flow-through water was used with a flow rate of 0.5 l h^{-1} through each 13 l beaker, and freshly mixed water was refilled every second day.

Samples of prebranchial haemolymph were collected from the ventral side of the abdomen, after washing with ethanol (70%). The soft part of the abdomen was pierced with a hypodermic needle (25 × 0.6 mm), and after the first drop of haemolymph was wiped away, 150 μl samples were collected with a micropipette. Following sampling, the haemolymph of the test animals was allowed to clot by keeping the crayfishes in air for 15 min in order to minimize an unrecorded haemolymph loss. In experiment 1, plasma and haemocytes were separated by dropping 300 μl haemolymph in a preweighed formaldehyde solution (3.3 mol l^{-1}) to prevent disintegration of the haemocytes. Each sample was then reweighed and centrifugated at 700 g for 10 min (Beckman J-21C), and the haemocytes were washed three times with formaldehyde before being suspended in 0.1 M HCl. For measuring Ca^{2+}, Na^{+}, and K^{+}, amounts of 100 μl haemolymph or 150 μl plasma-formaldehyde solution were diluted in 10 ml 0.1 M HCl. Measurements were made with an atomic absorption spectrophotometer (Unicam SP 1900). $LaCl_3$ was added to the Ca^{2+} samples to prevent binding to phosphate and organic material. For the K^{+} measurements, Na^{+} was added to the standard solutions to reach a comparable concentration between these and the samples. 50 μl haemolymph and 100 μl plasma-formaldehyde solution were used for Cl^- determinations, made within four hours with an automatic chloride titrator unit (Radiometer PHM 28).

In experiment 1, an unpaired Students t-test was used to determine whether differences in the concentrations of plasma and the haemocyte fraction before and after acid exposure were significant. The F-test was used to test the equality of variances (SAS Institute Inc., 1982). In experiments 2 and 3, differences between means of the control groups of treatment at day 0, were tested by an unpaired Students t-test. Analysis of variance, linear model (SAS Institute Inc., 1982), was used to test the significance of differences between changes in actual ion contents from day 0 and the following days of exposure. Least-square of means were calculated for each level of treatment.

Results

Experiment 1 was performed to determine the ratio between the ion content in haemocytes and whole haemolymph of *Astacus astacus,* however, total haemocyte count was not determined. Haemocyte K^+ was found to be an important component of the total K^+ content of haemolymph, and ca. 9% of the total K^+ content of haemolymph originated from the haemocytes in neutral water, while less than 1% of the total Na^+ content originated from the haemocytes (Table 1).

Acid exposure at pH 3.7 for three days reduced the haemocyte fraction of K^+ significantly ($p < 0.05$), but the haemocyte Na^+ fraction was not affected. Contradictory, plasma Na^+ was reduced by acid treatment ($p < 0.05$), while plasma K^+ was not, and in the same experiment plasma Ca^{2+} rose and plasma Cl^- decreased ($p < 0.05$ for both, Table 1). Total haemolymph concentrations of Na^+, Ca^{2+}, K^+, and Cl^- were significantly affected at pH 4.0 after 11 days (Fig. 1a–d). To test the significance of differences in concentrations between days and pH levels, analsysis of variance was used. Na^+ and Cl^- decreased at a constant rate during the experimental period, Na^+ slightly more rapidly than Cl^-. The changes in K^+ and Ca^{2+} concentrations were small; significant losses were recorded on day 11 at pH 4.0 ($p < 0.05$ and $p < 0.01$, respectively). A significant difference in Ca^{2+} concentration between the control group and the other two groups at day 0 ($p < 0.05$) was also found. Although the observed changes in haemolymph ion content were less than 30% at pH 4.0 after 11 days exposure, a significant loss of Na^+ and Ca^{2+} were recorded at pH 5.0 ($p < 0.01$ for both), however, the losses were

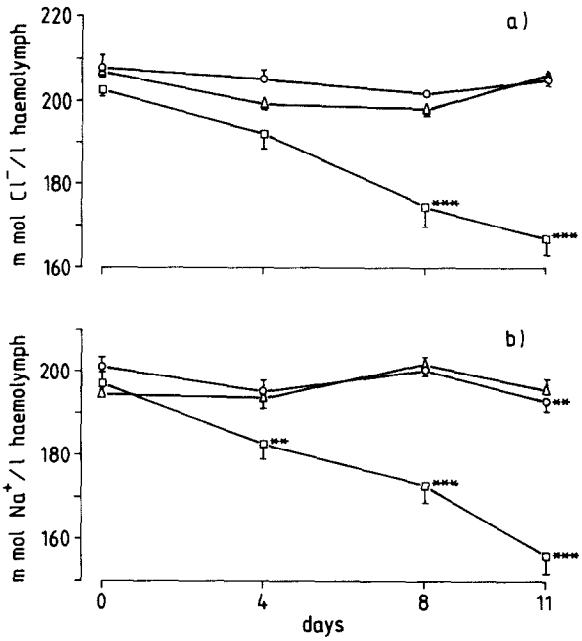

Fig. 1a and b. Haemolymph concentrations of Cl^- and Na^+ in *Astacus astacus* exposed to acid water, △—△ pH = 6.8 (control group), ○—○ pH = 5.0, □—□ pH = 4.0. Means ± S.E.M. N = 8. Asterisks indicte mean changes from day 0 significantly different from the control group by analysis of variance, least square-means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. When no vertical bar is present, the dot representing the mean is larger than S.E.M.

Table 1. Ion content in the plasma and haemocyte fractions of haemolymph of *Astacus astacus* before and after 3 days of exposure to pH 3.7. Means ± S.E.M., N = 8.

Fraction	Before exposure (pH 6.8)		After exposure for 3 days to pH 3.7	
	μmol/g haemolymph	% of total haemolymph	μmol/g haemolymph	% of total haemolymph
Na^+				
Plasma	191.1 ± 2.5	99.06	142.8 ± 5.7*	98.99
Haemocyte	1.82 ± 0.23	0.94	1.45 ± 0.45	1.01
K^+				
Plasma	3.45 ± 0.13	91.27	3.38 ± 0.23	97.97
Haemocyte	0.33 ± 0.11	8.73	0.07 ± 0.02*	2.03
Ca^{2+}				
Plasma	8.94 ± 0.33	–	11.00 ± 0.87*	–
Cl				
Plasma	198.5 ± 1.9	–	186.8 ± 4.2*	–

* Indicates means significantly different ($p < 0.05$) from corresponding pre-treatment value by unpaired *t* test.

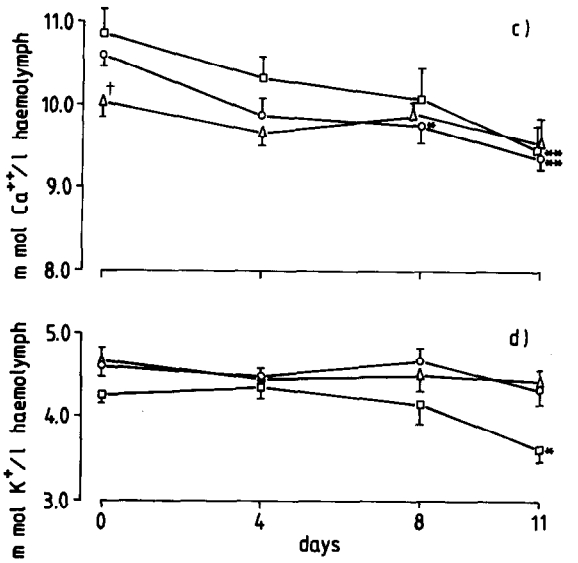

Fig. 1c and d. Haemolymph concentrations of Ca^{2+} and K^+ in *Astacus astacus* exposed to acid water, △—△ pH = 6.8 (control group), ○—○ pH = 5.0, □—□ pH = 4.0. Means ± S.E.M. N = 8. Asterisks indicate mean changes from day 0 significantly different from the control group by analysis of variance, least-square means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. † indicate that the mean of the control group is significantly different ($p < 0.05$) from the means of the other two groups on day 0 by unpaired *t* test. When no vertical bar is present, the dot representing the mean is larger than S.E.M.

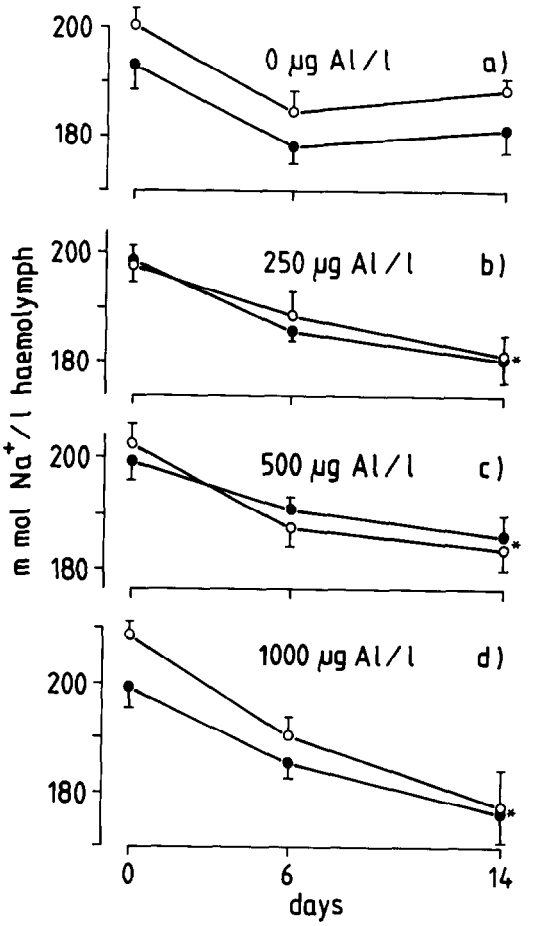

Fig. 2a–d. Haemolymph concentrations of Na^+ in *Astacus astacus* ●—● and *Pacifastacus leniusculus* ○—○ during exposure to different concentrations of aluminium in water at pH 5.0. Means ± S.E.M., N = 4. Asterisks indicate mean changes of both species from day 0, significantly different ($p < 0.05$) from the control group (0 µg Al^{3+}/l) by analysis of variance, least-square means.

smaller than at pH 4.0. The constant concentrations of the control group indicate that neither repeated sampling, nor starvation during the experimental period had any significant effect on the measured ion concentrations.

In order to study the effect of Al^{3+} on the haemolymph ion content of *A. astacus* and *P. leniusculus*, both species were exposed to four different concentrations of Al^{3+} (0, 250, 500, and 1 000 µg Al^{3+} l^{-1}) at pH 5.0 for 14 days. Haemolymph was sampled at neutral pH before the addition of Al^{3+} (day 0), and at the 6th and 14th days (Fig. 2a–d). Changes in ion concentrations from day 0 to days 6 and 14 were compared between the control group and the experimental groups, and after 14 days significant reductions of haemolymph Na^+ were noted in all groups treated with aluminium ($p < 0.05$).

There was no significant difference on any day between the two species (Fig. 2a–d), nor was there any significant difference between the Na concentrations of the four different groups at day 0.

Discussion

Studies by Höglund & Härdig (1969) and Neville (1979) show that a clear distinction between the effects of low pH and a raised level of free carbon dioxide, due to acidification, must be made. An increase of the carbon dioxide in the ambient water may induce a hypercapnic acidosis and affect haemolymph ion content (Dejours & Armand, 1982). In the experimental water, used in the present study, the calculated value for the maximal pCO_2 was less than 2.5 mm Hg, which indicates that the changes in haemolymph ion content were not due to high pCO_2 of the media.

A comparison of ion concentrations in haemocytes and plasma shows that haemocyte K^+ constitutes a considerable part of the total amount of haemolymph K^+. The total haemocyte count of haemolymph in crustaceans can be affected by several factors such as temperature, cuticle damages, and infections (Söderhäll, 1982; Smith & Söderhäll, 1983), and therefore care has to be taken when evaluating changes of K^+ concentrations in samples of total haemolymph. The constant amount of Na^+ in the haemocyte fraction in experiment 1, indicates that the decrease of K^+ in the haemocyte fraction after acid exposure is due to a loss of intracellular K^+ and not a loss or lysis of haemocytes.

Acid exposure of fish has been reported to produce a loss of intracellular K^+ from heart tissue and muscle tissue to the plasma fraction (Fugelli & Vislie, 1980, and McDonald & Wood, 1981, respectively), which corresponds to the loss of haemocyte K^+ found here. It is suggested that this intracellular loss is a result of K^+ acting as a regulator of the cell volume, and caused by extracellular ion losses (Fugelli & Vislie, 1980). The non-linear decrease of haemolymph K^+, recorded at pH 4.0, indicates a net loss of K^+ similar to that reported for *Salmo gairdneri* by McDonald & Wood (1981). A reduced net accumulation rate of K^+ is also found in developing eggs of *A. astacus* exposed to acid water at pH 4.9 (Appelberg, 1984).

A reduction of plasma concentrations of Na^+ and Cl^- during acid stress has previously been reported for several fish species (Packer & Dunson, 1970; Leivestad *et al.*, 1976; McWilliams, 1980; McDonald & Wood, 1981), and for crayfish by Morgan & McMahon (1982) and McMahon & Morgan (1983). This loss is probably due to a reduced uptake rate of Na^+ and Cl^- over the gills, resulting largely from a disturbance of the Na^+/H^+ and Cl^-/basic anion exchange mechanisms, with a possible contribution from an increased passive efflux of Na^+ (Morgan & McMahon, 1982). The reduction of haemolymph Cl^- may also be related to the actual decrease in haemolymph Na^+, since the uptake rate of Cl^- is dependent on haemolymph Na^+ concentration (Shaw, 1964).

Morgan & McMahon (1982), who recorded a drastic increase of haemolymph Ca^{2+} in *Procambarus clarkii* when exposed to water at pH 3.8, suggest that exoskeletal carbonate is used to buffer an internal acidosis. In the present study, plasma Ca^{2+} rose in experiment 1, when exposed to pH 3.7, while there was a slight decrease in total haemolymph Ca^{2+} in experiment 2, when exposed to pH 4.0 and 5.0. It is possible that the difference in pH levels may influence the suggested buffering by $CaCO_3$ of the haemolymph, but the rate of Ca^{2+} loss from haemolymph may also be attributed to differences within the intermoult stage, with is found to influence the Ca^{2+} metabolism (Greenaway, 1972).

Despite a significant loss of Na^+ within 14 days, at an aluminium concentration of 250 $\mu g\ l^{-1}$ at pH 5.0, the present effects on both *A. astacus* and *P. leniusculus* are small in comparison with those reported for fish. Driscoll *et al.* (1980), recorded a 50% survival time of 115 h of brook trout, *Salvelinus fontenalis,* at pH 5.2 and 0.42 mg $Al^{3+}\ l^{-1}$, and Muniz & Leivestad (1980b), recorded toxic effects of 190 $\mu g\ Al^{3+}\ l^{-1}$ at pH 5.0 to brown trout *Salmo trutta.* However, Havas & Hutchinson (1982) found that high concentrations of aluminium (20 $mg\ l^{-1}$) is highly toxic to smaller crustaceans, and it is possible that a higher aluminium concentration would have caused a more drastic response in the present study. The decrease in Na^+ content in all groups (Fig. 2) between days 0 and 6, seems to be a true effect of lowered pH at day 0 only.

Differences in ion composition of experimental waters influences comparisons between different studies, since it is known that the uptake rate of Na^+, Cl^-, and Ca^{2+} in crayfish to some extent depends on the concentrations in the medium (Shaw, 1959, 1960; Greenaway, 1974). However, the changes in haemolymph ion concentration reported for *A. astacus* in the present study, in general agree with the results of Morgan & McMahon (1982) and McMahon & Morgan (1983) obtained for *Procambarus clarkii* and *Orconectes rusticus* during acid exposure.

The present study indicates that also slightly acid water (pH 5.0) may, in the long run, disturb the ion regulation in *A. astacus* and preliminary studies gave similar effects of acid stress on *P. leniusculus.* The absence of these species in acid waters (Svärdson, 1974), is possibly due to a complex of factors; changes of habitat, predators, inter-specific competition, and food organisms. As judged from the present findings, disturbance of the ion regulation, mainly depending on low pH, and to lesser extent possibly also aluminium, may contribute to this. More susceptible stages in the life history, such as

reproduction and moulting, seem to be more critical during acid exposure compared to the intermoult, adult, crayfish used in the present study (Malley, 1980; Appelberg, 1984).

Summary

1. Haemolymph concentrations of Na^+, K^+, and Cl^- decreased, whereas the concentration of Ca^{2+} fluctuated in *Astacus astacus* L. during exposure to soft, acid water. Weak acid stress, pH 5.0, reduced the content of both Na^+ and Ca^{2+} in the haemolymph within 11 days.
2. After exposure to pH 3.7 for 3 days the content of the haemocyte K^+ was reduced.
3. Exposure to 250 μg Al^{3+} l^{-1} in water at pH 5.0 for 14 days, reduced the haemolymph content of Na^+ in *Astacus astacus* and *Pacifastacus leniusculus*.

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