The Journal of Experimental Biology 208, 951-959
Published by The Company of Biologists 2005
doi:10.1242/jeb.01426

Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*

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Accepted 1 December 2004

Summary

Daphnia **are highly sensitive to sodium metabolism disruption caused by aquatic acidification and ionoregulatory toxicants, due to their finely balanced ion homeostasis. Nine different water chemistries of varying pH (4, 6 and 8) and calcium concentration (0, 0.5 and 1 mmol l^{-1}) were used to delineate the mechanism of sodium influx in *Daphnia magna*. Lowering water pH severely inhibited sodium influx when calcium concentration was high, but transport kinetic analysis revealed a stimulated sodium influx capacity (J_{max}) when calcium was absent. At low pH increasing water calcium levels decreased J_{max} and raised K_m (decreased sodium influx affinity), while at high pH the opposite pattern was observed (elevated J_{max} and reduced K_m). These effects on sodium influx were mirrored by changes in whole body sodium levels. Further examination of the effect of calcium on sodium influx showed a severe inhibition of sodium uptake by 100 μmol l^{-1} calcium gluconate at both low (50 μmol l^{-1}) and high (1000 μmol l^{-1}) sodium concentrations. At high sodium concentrations, stimulated sodium influx was noted with elevated calcium levels. These results, in addition to data showing amiloride inhibition of sodium influx (K_i=180 μmol l^{-1}), suggest a mechanism of sodium influx in *Daphnia magna* that involves the electrogenic $2Na^+/1H^+$ exchanger.**

Key words: acid precipitation, soft water, hardness, osmoregulation, invertebrate, *Daphnia magna.*

Introduction

The homeostatic control of ion balance is a major metabolic cost of life in freshwater. Faced with the continuous loss of ions from the concentrated body tissues to the dilute external milieu, freshwater animals have developed a number of physiological mechanisms to ensure a constant internal ion status. One such mechanism, common to all freshwater osmoregulators, is active ion uptake (Potts and Parry, 1964).

The mechanism of sodium uptake in freshwater organisms has been extensively investigated. Sodium transport across the gill of freshwater-adapted crabs is thought to be powered by active proton extrusion (for a review, see Kirschner, 2004). An apical V-type H^+-ATPase provides an electrochemical gradient for the passage of sodium ions from freshwater into the gill cell *via* a sodium channel. Sodium is consequently driven across the basolateral surface by the ATP-dependent sodium–potassium pump (Na^+/K^+-ATPase). This is similar to the mechanism of uptake in freshwater fish (Evans et al., 1999). Conversely in freshwater-adapted euryhaline crayfish, apical sodium transfer is likely achieved by an apical sodium–proton exchange mechanism, where the extrusion of protons and the uptake of sodium are mediated by the same transport moiety (Kirschner, 2004). In the freshwater cladoceran *Daphnia magna* it is known that sodium uptake is saturable, indicating a specific transport mechanism is involved (e.g. Stobbart et al., 1977; Potts and Fryer, 1979; Glover et al., 2005). Furthermore sodium uptake is reduced as pH is decreased, suggesting that sodium uptake may be linked to proton excretion (Potts and Fryer, 1979).

Proton-linked sodium uptake likely explains the high sensitivity of freshwater animals to aquatic acidification. Fish, molluscs and crustaceans have disappeared from many fresh waters as a result of acid precipitation (e.g. Leivestad et al., 1976). The mechanism behind such mortalities appears to be the breakdown in sodium ion regulation (see Vangenechten et al., 1989; Wood, 1989). In freshwater fish, mortality in acid waters appears to be mediated by an inhibition of sodium influx, and an enhanced sodium efflux (Wood, 1989). The influx inhibition is likely a consequence of acid interference with sodium transport processes, be it a direct competition between protons and sodium ions for uptake (Wood, 1989), or an indirect effect caused by the loss of the outward proton gradient that drives inward sodium flux (Lin and Randall, 1995). In fish, the presence of calcium in acid waters appears to protect against sodium depletion. Raising calcium levels in laboratory experiments is believed to replace the calcium leached from tight junctions by enhanced acidity (see Wood, 1989). This calcium addition reduces junction permeability,

decreases paracellular sodium efflux and favourably influences whole body sodium status.

Mechanistic knowledge of sodium transport pathways in the highly sensitive freshwater crustacean *Daphnia magna* will contribute greatly to our understanding of how these organisms respond to environmental stressors such as acid precipitation, and also to environmental metal contamination. Silver, for example, inhibits sodium uptake pathways and thus causes mortality at extremely low concentrations (Bianchini and Wood, 2003). The inability to replace lost sodium rapidly depletes whole body sodium concentrations and results in mortality. Acute median lethal toxicity values of less than 1 μg l^{-1}, make daphnids the most sensitive of all freshwater animals to environmental silver (for a review, see Wood et al., 2002). Mechanistic knowledge of sodium uptake pathways would enhance our understanding of the mode of toxicity of silver and other metal toxicants that are likely to interfere with this process (e.g. copper).

In this study a transport kinetic approach has been utilised to determine the effects of hydrogen and calcium ions on sodium influx in *Daphnia magna*. This type of approach is beneficial in that changes in parameters derived from Michaelis–Menten analysis may provide mechanistic information. Alterations in transport affinity and/or transport capacity may be characteristic of either competitive or non-competitive interactions at the transport site, or a combination of both (Cornish-Bowden, 1979). The nature of acid and calcium interactions with sodium influx will provide insight into mechanisms of sodium influx, and will have implications for the response of freshwater crustaceans to anthropogenic modifications of the natural environment, including metal pollution and acid precipitation.

Materials and methods

Daphnia culture

A laboratory population of *Daphnia magna* was established from a culture obtained from Aquatic Research Organisms (ARO strain; Hampton, NH, USA), and maintained under constant light (16 h:8 h L:D), temperature (20–22°C), and water chemistry (synthetic Lake Ontario water: 1 mmol l^{-1} $CaCO_3$, 0.15 mmol l^{-1} $MgSO_4.7H_2O$, 0.6 mmol l^{-1} NaCl, pH 8) conditions. This culture medium was reconstituted from reverse osmosis water. For all experiments 7–8-day-old *Daphnia* (~1 mg wet mass) were used. *Daphnia* used in the experiments were isolated within several hours of birth to ensure a similar moulting stage at the time of experimentation.

Experimental manipulations

The effect of water calcium and pH on *Daphnia* sodium influx was determined at three calcium concentrations (0, 0.5 and 1 mmol l^{-1}; as $CaSO_4$), and at three pH levels (4, 6 and 8). Experimental media were all prepared from deionised water (>17.5 MΩ cm; Barnstead Nanopure II, Dubuque, IA, USA). To permit kinetic analysis of sodium uptake at each of these nine water chemistries, five sodium concentrations (50, 150, 300, 750 and 1500 μmol l^{-1}; as NaCl) were analysed. This resulted in a total of 45 experimental chambers (100 ml of solution in an acid-washed 250 ml glass beaker; Pyrex). To each chamber ^{22}Na (~1 kBq ml^{-1} as NaCl; Perkin Elmer, Boston, MA, USA) was added as a marker of sodium influx. Adjustment of pH (0.1 N KOH or 0.1 N HNO_3) was performed ~16 h prior to experiment commencement, with a final adjustment of pH within 3 h of daphnid introduction. Six *Daphnia* were added to each chamber, and influx was monitored over 1 h. The high solution to biomass ratio, the use of experimental water reconstituted from deionised water, and the short flux measurement duration sought to minimise the contribution of organic carbon, which could potentially complicate sodium metabolism (Glover et al., 2005).

To further delineate the actions of calcium on sodium influx kinetics, a follow-up study utilising a similar protocol was employed. The calcium concentration-dependence of sodium uptake was examined over a wide range of calcium concentrations (0, 50, 100, 500, 1000, 5000 μmol l^{-1}; as calcium gluconate), in the presence of low (50 μmol l^{-1}) or high (1000 μmol l^{-1}) sodium water concentrations. This experiment was conducted with two sodium salts: sodium chloride and sodium gluconate. The use of the gluconate salt introduced sodium into solution with an impermeant anion, and thus permitted an additional analysis of the influence of Cl^- on sodium influx. These experiments used identical radiotracer specific activities (~1 kBq ml^{-1} as NaCl; Perkin Elmer), water volumes (100 ml), daphnid numbers (6), and influx times (1 h), to that described above, with a pH ~6.

The effect of amiloride (*N*-amidino-3,5-diamino-6-chloro-pyrazinecarboxamide hydrochloride; Sigma, St Louis, MO, USA) on sodium influx was examined at two sodium concentrations (50 μmol l^{-1} and 300 μmol l^{-1} as NaCl). Amiloride (10, 50, 100, 500, 1000, 5000 or 10 000 μmol l^{-1}) was added from a concentrated stock solution to 50 ml of an appropriate sodium solution. Five *Daphnia* were added to each test chamber, and influx was determined from uptake of radiotracer (^{22}Na; ~1 kBq ml^{-1} as NaCl; Perkin Elmer) over 15 min. This time was chosen to minimise the acutely toxic effects of the amiloride exposure. In an additional treatment, daphnids were pre-exposed to the highest amiloride concentration (10 000 μmol l^{-1}) for 15 min, rinsed in synthetic Lake Ontario water for ~1 min, then added to amiloride-free experimental chambers containing radiolabelled sodium for 15 min.

Sodium influx determination and whole body sodium measurement

Daphnia from all experimental treatments were analysed for sodium influx in an identical manner. Following removal from experimental chambers, daphnids were rinsed (~10 s) in a high sodium displacement solution (~1 mol l^{-1} NaCl), with two subsequent rinses (~15 s each) in deionised water. Animals were blotted dry, weighed (UMT2, Mettler-Toledo, Greifensee, Switzerland; 0.001 mg precision), and counted for

γ-activity (Canberra-Packard, Minaxi Auto-gamma 5000, Meridian, CT, USA). Sodium influx was calculated from the equation J_{in}=c.p.m./(*SAmt*), where c.p.m. is the γ counts per minute in the daphnid, *SA* is the specific activity of the exposure water (c.p.m. μequiv^{-1}), *m* represents the daphnid wet mass (in g, corrected for trapped carapace water by multiplying by 1.25; Stobbart et al., 1977), and *t* is the time of exposure in h. This resulted in a sodium influx expressed as μequiv g wet mass h^{-1}.

Daphnids from the combined pH/calcium experimental protocol were also analysed for whole body sodium content. Individual animals were digested in 50 μl of concentrated H_2SO_4 (trace metal grade; Fisher, Nepean, ON, Canada), before being diluted with deionised water to an appropriate concentration for analysis *via* flame atomic absorption spectrophotometry (220FS, Varian, Palo Alto, CA, USA). Whole body sodium concentrations were calculated as the sodium concentration in the daphnid, per unit wet mass, again accounting for trapped carapace water.

Data analysis

Data points have been routinely expressed as means ± S.E.M. (*N*=number of individuals). Statistical significance was determined by one-way or two-way analysis of variance (ANOVA), followed by *post-hoc* LSD analysis (Statistica 5.1; Statsoft, Tulsa, OK, USA).

Kinetic analysis of sodium influx was modelled using the Michaelis–Menten equation, $J_{in}=J_{max}[Na^+]/K_m+[Na^+]$, where J_{max} is the maximal rate of sodium influx and K_m is the sodium concentration at which sodium influx is half maximal. Values of J_{max} and K_m were taken directly from plots of sodium influx *versus* sodium concentration using SigmaPlot (ver. 8.0.2; SPSS, Inc.). Each curve represents the sodium influx of 5–6 individuals at each of 5 sodium concentrations.

Differences between kinetic parameters were determined by *t*-tests, using the parameter and its S.E.M. as determined by best fit Michaelis–Menten analysis (Motulsky, 1998). A conservative approach was taken by treating each sodium concentration, as opposed to each individual, as a single value. Consequently each pairwise comparison was assessed with 8 degrees of freedom [2(*N*–1)]. This conservative approach compensated for the lack of multiple comparison corrections, which were considered inappropriate due to the inflated chance of type II error (Perneger, 1998).

Results

The effect of calcium and pH on sodium influx is shown in Fig. 1. For all curves sodium influx conformed to Michaelis–Menten kinetics. Sodium influx increased as external sodium concentration was raised, until saturation was observed at high sodium levels. The single exception to this pattern was observed at a calcium level of 1 mmol l^{-1} and a pH of 4 (Fig. 1C). In this water chemistry, sodium influx in *Daphnia* was approximately linear with respect to sodium concentration over the range of sodium levels tested. Consequently, kinetic parameters could not be calculated for this treatment.

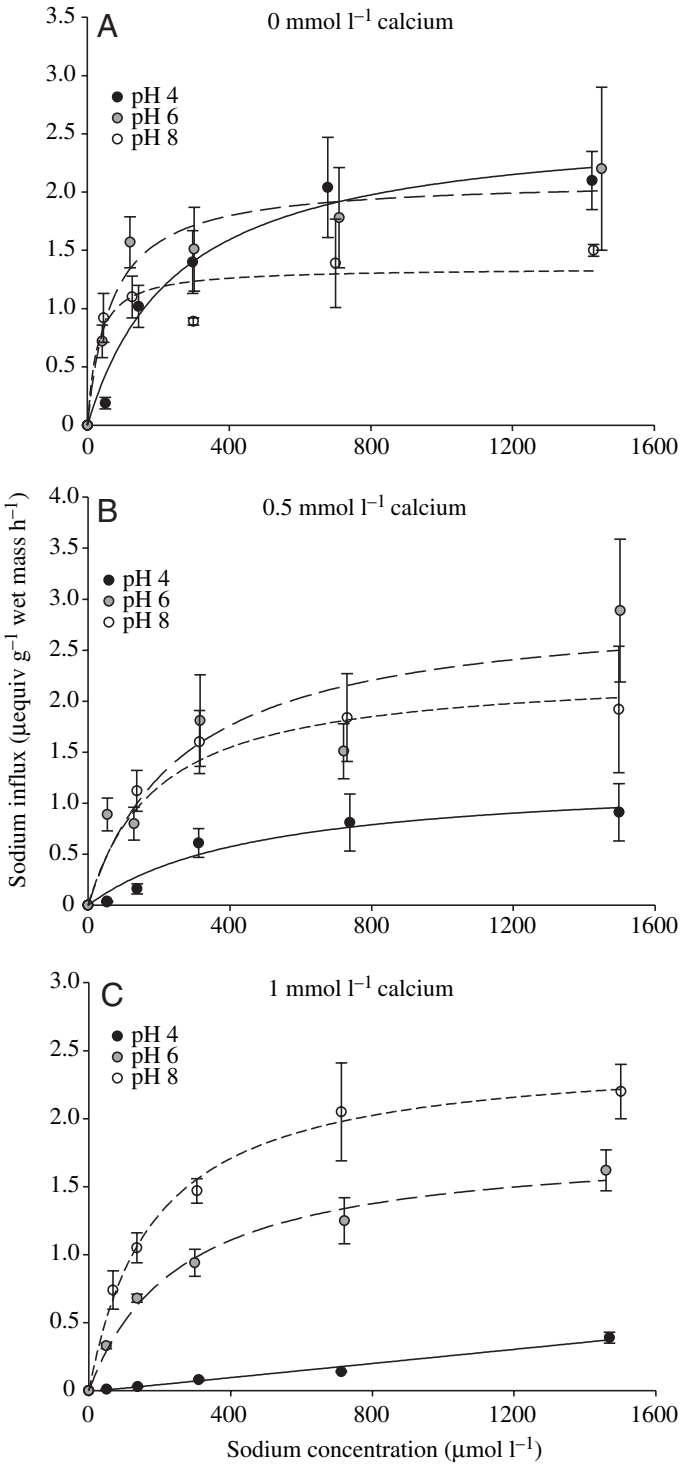

Fig. 1. Sodium influx (μequiv g^{-1} wet mass h^{-1}) in *Daphnia magna* as a function of external sodium concentration, pH (4, 6 or 8) and calcium concentration (A, 0 mmol l^{-1}; B, 0.5 mmol l^{-1}; C, 1 mmol l^{-1}). Each plotted point represents the mean ± S.E.M. of 5–6 daphnids exposed under experimental conditions described in Materials and methods.

The kinetic parameters illustrated in Fig. 2 were derived from the curves shown in Fig. 1. The maximal rate of sodium influx (J_{max}) was strongly influenced by calcium level and pH of the ambient water (Fig. 2A). In the absence of calcium, decreasing pH (increasing proton concentration) raised J_{max} from a value of 1.2 ±0.09 μequiv mg^{-1} wet mass h^{-1} at pH 8 to 2.11±0.32 μequiv mg^{-1} wet mass h^{-1} at pH 6. The opposite effect was observed when calcium was high (1 mmol l^{-1}), with decreasing pH inhibiting maximal sodium influx. Low pH (4) also significantly reduced J_{max} at intermediate calcium

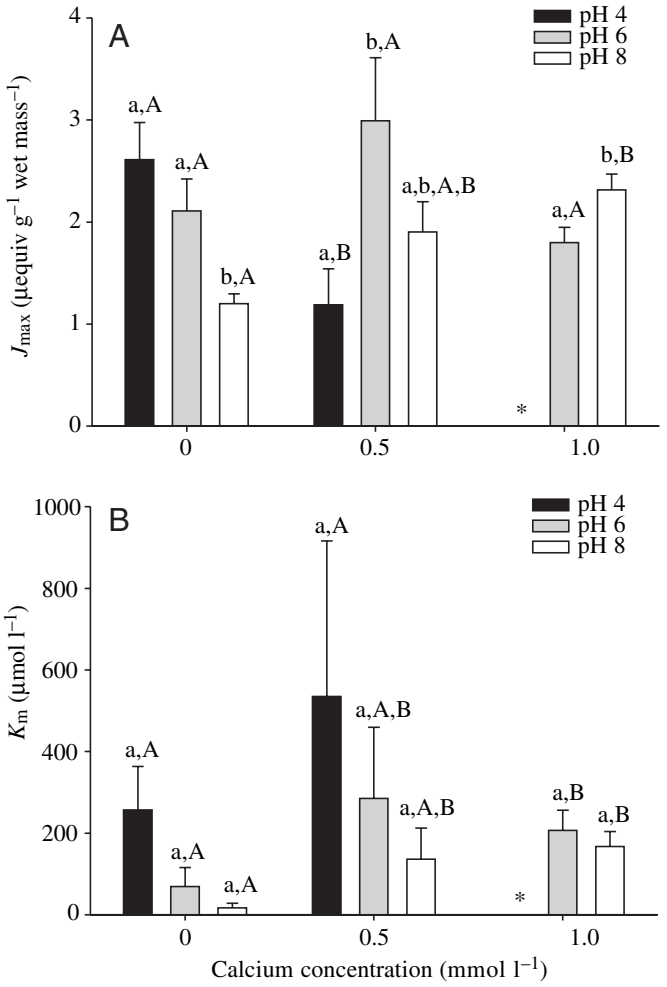

Fig. 2. Effect of external pH (4, 6 or 8) and calcium (0, 0.5 or 1 mmol l^{-1}) on *Daphnia magna* sodium transport capacity (A; J_{max}, μequiv g^{-1} wet mass h^{-1}) and affinity (B; K_m, μmol l^{-1}). Plotted points represent parameters calculated directly from the plots illustrated in Fig. 1A–C, using SigmaPlot (version 8.0.2; SPSS, Inc.). Missing data points (*) were those that did not conform to Michaelis–Menten kinetics, and were thus excluded. Bars sharing lowercase letters are not significantly different compared to other pH treatments within each calcium concentrations (i.e. effect of pH), whereas bars sharing uppercase letters are not significantly different compared to similar pH treatments at different calcium concentrations (i.e. effect of calcium). Statistical significance (P<0.05) was calculated as described in Materials and methods. Data points with asterisks were excluded from statistical comparison.

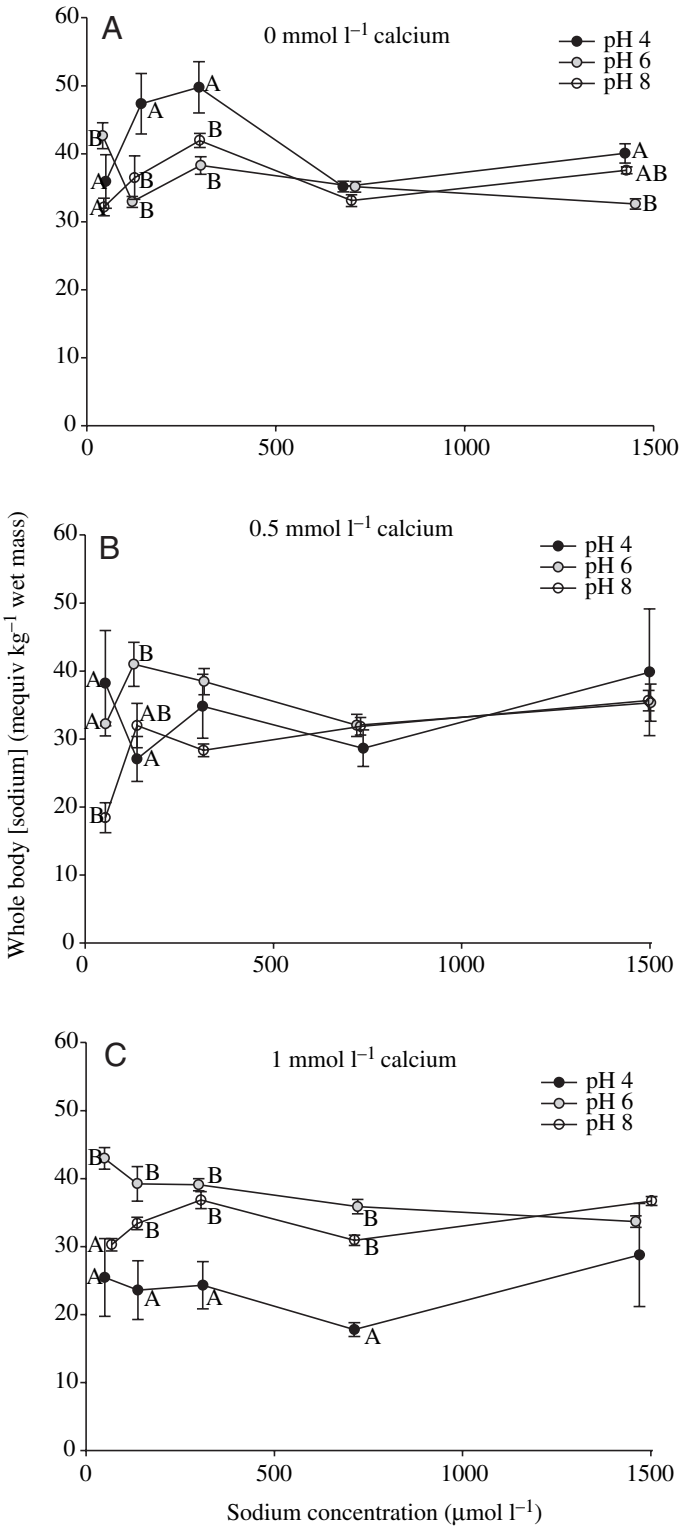

Fig. 3. Whole body sodium (mequiv kg^{-1} wet mass) in *Daphnia magna* as a function of external sodium concentration, pH (4, 6 or 8) and calcium (A, 0 mmol l^{-1}; B, 0.5 mmol l^{-1}; C, 1 mmol l^{-1}). Each plotted point represents the mean ± S.E.M. of 5–6 daphnids exposed under experimental conditions described in Materials and methods. Data points sharing letters are not significantly different (P<0.05) from similar points within each sodium concentration, as determined by two-way ANOVA.

concentrations (0.5 mmol l^{-1}). Within pH treatments, calcium exerted significant actions on sodium influx capacity. At low pH elevated calcium levels inhibited sodium influx. At high pH increased calcium levels stimulated J_{max} with a sodium influx of 1.20±0.09 μequiv mg^{-1} wet mass h^{-1} recorded for daphnids exposed to pH 8 and 0 mmol l^{-1} calcium, compared to a value of 2.32±0.16 μequiv mg^{-1} wet mass h^{-1} for those exposed to pH 8 and 1 mmol l^{-1} calcium in the ambient water.

Effects of calcium and pH on sodium influx affinity (K_m) were less prevalent (Fig. 2B). Within calcium concentrations, pH had no influence on sodium uptake affinity. Comparisons between different calcium levels at a constant pH revealed an enhanced K_m (decreased sodium influx affinity) with increased calcium levels at pH 6 and 8. A threefold increase in K_m was observed at pH 6 when calcium was increased from 0 to 1 mmol l^{-1} (69±47 *vs*. 207±49 μmol l^{-1}), while at pH 8, similar increases in calcium raised the K_m tenfold (17±11 *vs*. 167±37 μmol l^{-1}).

The influence of calcium and pH on whole body sodium content reflected the effects observed on sodium influx (Fig. 3A–C). At low pH, elevated sodium status was observed at low calcium levels. Conversely at low pH, significantly reduced whole body sodium contents were associated with high waterborne calcium levels. Whole body sodium concentrations at pH 4 and 1 mmol l^{-1} calcium were in the order of 20–25 mg kg^{-1} wet mass, approximately 50–75% of whole body sodium content at pH 8 and 1 mmol l^{-1} calcium.

The effect of calcium on sodium influx was investigated further (Fig. 4). At both low (50 μmol l^{-1}) and high (1000 μmol l^{-1}) sodium levels there was no statistical difference between the two sodium salts tested. At low sodium levels (Fig. 4A), calcium inhibited sodium influx. Sodium influx was especially sensitive to inhibition by low levels of calcium. The addition of 100 μmol l^{-1} calcium decreased sodium (as gluconate) influx from 0.82±0.21 to 0.49±0.08 μequiv g^{-1} wet mass h^{-1}. Despite a 50-fold increase in calcium concentration, there was only minimal additional reduction in sodium influx.

At 1000 μmol l^{-1} sodium (Fig. 4B), calcium levels up to 100 μmol l^{-1} were again observed to inhibit sodium influx. This effect followed a similar pattern to that observed at 50 μmol l^{-1} with a maximal inhibition of 54% noted at a calcium concentration of 100 μmol l^{-1} for the sodium chloride experiment. This decrease was not, however, statistically significant. As calcium levels were raised further inhibition of sodium influx was not observed, and instead sodium influx rates were restored to control (calcium-free) levels.

Amiloride inhibited sodium influx at both low and high sodium concentrations in a dose-dependent manner (Fig. 5A). Maximal sodium influx inhibitions of 93% (at 50 μmol l^{-1} sodium) and 85% (at 300 μmol l^{-1} sodium) were reached at 5 mmol l^{-1} amiloride, with addition of higher amiloride concentrations having no further effect on sodium influx. Pre-treatment with amiloride resulted in similar sodium inhibition effects (not shown). A Dixon plot (Fig. 5B) was constructed for amiloride concentrations up to 5 mmol l^{-1} (maximal inhibition). From this figure the inhibition constant (K_i) for the effect of amiloride on sodium influx was calculated as 180 μmol l^{-1} amiloride. The amiloride concentration at which the two lines intersect is the inverse of the K_i (Cornish-Bowden, 1979).

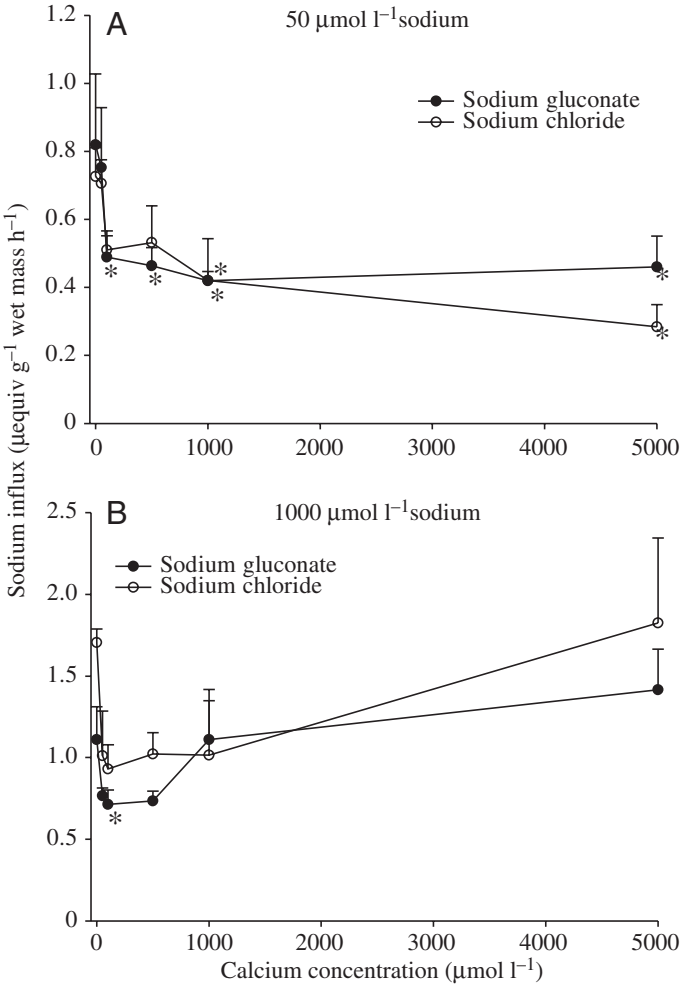

Fig. 4. Effect of external calcium concentration (0–5000 μmol l^{-1} calcium gluconate) on sodium influx (μequiv g^{-1} wet mass h^{-1}) in *Daphnia magna* at two different sodium concentrations (A, 50 μmol l^{-1}; B, 1000 μmol l^{-1}), and two different sodium salts (sodium chloride, white circles; sodium gluconate, black circles). Each data point represents the mean of 5–6 individuals. *Statistical significance (P<0.05) was determined by one-way ANOVA, and represents differences between the plotted point and the control (0 calcium). There were no significant differences in the calcium dependence of sodium influx between sodium chloride and sodium gluconate.

Discussion

Effect of pH on sodium influx in Daphnia magna *is calcium dependent*

Analysis of the transport kinetics of sodium influx in *Daphnia magna in vivo* reveals a complex pattern, highly influenced by external pH and calcium. The impact of acid

waters on sodium metabolism in aquatic life has been well documented. Inhibitory effects on sodium influx and exacerbated efflux lead to whole body sodium depletion, and potentially mortality (Vangenechten et al., 1989; Wood, 1989). In the present study, at 1 mmol l^{-1} calcium, sodium influx at pH 4 was almost completely inhibited (Fig. 1C). The saturable uptake kinetics observed in other water chemistries was eliminated, leaving a small, presumably diffusive, component of influx. In fish, the inhibitory effect of acid on sodium influx has been explained in terms of a competitive inhibition of Na^+ transport by H^+ (Wood, 1989), or a reduction in H^+ gradient that reduces proton efflux, and consequently the driving force for sodium influx (Lin and Randall, 1995). In high calcium conditions the observed changes in sodium influx are consistent with a sodium uptake mechanism linked to proton efflux, as suggested previously for Cladocerans (Potts and Fryer, 1979).

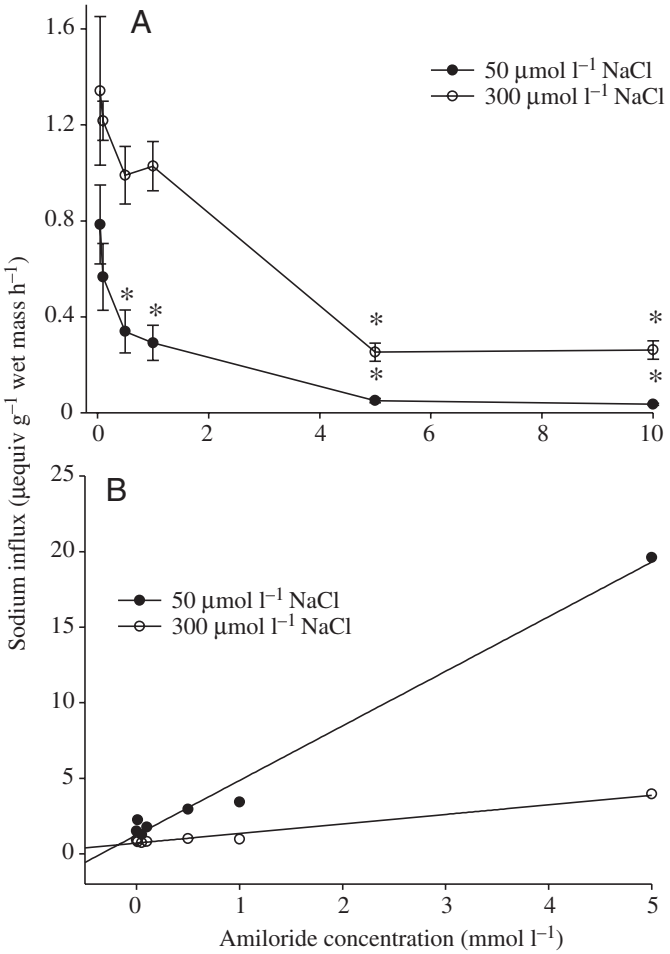

Fig. 5. Effect of amiloride (0–10 000 µmol l^{-1}) on sodium influx (µequiv g^{-1} wet mass h^{-1}) in *Daphnia magna* at two different sodium concentrations (50 or 300 µmol l^{-1}). *Statistical significance (P<0.05) was determined by one way ANOVA, and represents differences between the plotted point and the control (0 amiloride). The data represented in A were replotted in the Dixon plot (B). The K_i of amiloride inhibition of sodium influx was calculated as the inverse of the x-value of the intersection point of the two lines. Some data points at low amiloride concentrations were excluded for clarity, and the 10 000 µmol l^{-1} amiloride concentration was excluded from the Dixon plot owing to maximal inhibition being attained at 5000 µmol l^{-1} amiloride.

In the absence of calcium, pH had the opposite effect on sodium influx in *Daphnia* than that observed at 1 mmol l^{-1} calcium. Under calcium-free conditions increasing proton concentration from pH 8 to 4 had no significant effect on sodium transport affinity (K_m), yet stimulated sodium transport capacity (J_{max}). This suggests the actions of protons at low calcium, in contrast to its actions at high calcium, are not associated with a competitive interaction between sodium and protons at the active transport site, and instead may be characteristic of a sodium–proton exchange mechanism.

In several invertebrate tissues the presence of an electrogenic sodium–proton exchanger has been suggested by physiological (Ahearn and Clay, 1989; Shetlar and Towle, 1989), immunohistochemical (Kimura et al., 1994), molecular (Towle et al., 1997) and oocyte expression (Mandal et al., 2001) studies. This apical exchanger, unlike that found in vertebrate and prokaryotic systems, mediates the influx of two sodium ions in exchange for a single proton (for a review, see Ahearn et al., 2001). It is further distinct from these exchangers in that it exhibits strong calcium-dependence (Ahearn and Franco, 1990; Zhuang and Ahearn, 1996). This exchanger is capable of transporting calcium across the apical surface and thus performs a potentially important role in calcium homeostasis (Ahearn et al., 2004). It appears that calcium and sodium share the same transport site, as these ions reciprocally inhibit the others passage in a competitive manner (Ahearn and Franco, 1990). Such a mechanism is consistent with the findings herein, of reduced sodium influx at high calcium levels, due in part to a competitive interaction (effect on K_m).

Another important feature of this invertebrate electrogenic $2Na^+/1H^+$ exchanger is its cooperativity. A distinctive sigmoidal influx curve is generated as a function of increasing external sodium levels (Ahearn and Clay, 1989; Shetlar and Towle, 1989). In most invertebrate cell types and tissues where this antiporter has been proposed to be responsible for sodium uptake, Hill coefficients close to two are described (see Ahearn et al., 2001; Mandal et al., 2003), suggesting cooperativity of sodium binding. In *Daphnia* sodium influx was a hyperbolic, not a sigmoidal, function of external sodium concentration. In the current study, sodium influx was monitored over a range of sodium concentrations that are within a relevant range for a freshwater organism (0–1500 µmol l^{-1}). This range is considerably lower than that examined in previous investigations of uptake kinetics *via* this exchanger (range from ~2.5 up to 400 mmol l^{-1}). Previous studies have focussed on sodium uptake in epithelia that are routinely exposed to relatively sodium-enriched milieus (marine invertebrate gills, gut; Ahearn et al., 2001). Sodium concentrations examined in *Daphnia* therefore are at levels unlikely to generate sigmoidal uptake kinetics. Thus the role of a putative $2Na^+/H^+$ exchanger in facilitating sodium influx in *Daphnia* cannot be excluded. This does, however, suggest that in low sodium freshwaters the

transporter may function according to Michaelis–Menten kinetics, transporting a single sodium ion across the apical surface.

Cooperativity is generated by the existence of more than one sodium binding site. This has been demonstrated in freshwater prawn hepatopancreas by amiloride inhibition data showing two distinct binding sites *via* Dixon plot analysis (Ahearn and Clay, 1989). While Shetlar and Towle (1989) described a single inhibition constant of amiloride, suggesting a single sodium binding site, they also noted that the square of the amiloride concentration gave a better fit to the sodium influx inhibition data. Thus they suggested the presence of two amiloride binding sites. Similarly, while a single K_i of 180 μmol l^{-1} was described for the effect of amiloride on sodium influx in the present study, the square of the amiloride concentration was a better fit (r^2 0.9878 *vs.* 0.9924 for the low sodium treatment). Based on the interpretation of Shetlar and Towle (1989), the results presented here could also be suggestive of a two-binding-site model, and again could support the existence of a $2Na^+/1H^+$ exchanger.

While sodium concentrations used in this experiment were likely insufficient to generate cooperativity, proton levels may have resulted in cooperative effects. The non-competitive stimulation of sodium transport observed in the absence of calcium and the presence of high proton concentration could be evidence of such an effect. Proton binding to one of the putative sodium binding sites may have acted to facilitate sodium binding to the other sodium binding site, thus promoting increased sodium influx, in a cooperative manner. In the presence of calcium (1 mmol l^{-1}) such a mechanism may not exist, due to the ability of calcium to block the sodium transport site.

Calcium may both inhibit and stimulate sodium influx

As discussed above, the competitive effects of calcium on sodium influx appear to fit a mechanism of sodium influx that involves the invertebrate electrogenic $2Na^+/1H^+$ exchanger. The effect of calcium on sodium influx in *Daphnia magna* over this relatively small range of calcium levels (0–1 mmol l^{-1}) was extended to a larger range of calcium concentrations (0–5 mmol l^{-1}). At a low sodium concentration (50 μmol l^{-1}) the dose-dependent inhibition of calcium was prominent, clear-cut and occurred at a relatively low external calcium concentration (100 μmol l^{-1}). At a higher sodium concentration (1000 μmol l^{-1}), inhibition was also discerned up until a calcium level of 100 μmol l^{-1}. Thereafter, however, increasing calcium stimulated sodium influx. This suggests the possibility of a calcium-stimulated sodium uptake pathway that initially negates, then supersedes the inhibitory actions of calcium at lower sodium and calcium levels. The presence of apical sodium/calcium exchange has been described in invertebrate tissues (Zhuang and Ahearn, 1996). This transporter would likely only be active when calcium levels are high, and may serve as a mechanism for regulating intracellular calcium. As calcium is transported out of the cell, sodium would move into the cell, thus influx stimulation would be observed. As this mechanism only appears to operate at relatively high sodium levels it suggests this pathway may have a comparatively low affinity for sodium, and thus may be of limited physiological relevance as a route of sodium influx in *Daphnia*.

The results of the calcium inhibition study also showed that there was no effect of anion on sodium influx. Sodium influx data were statistically identical when either chloride or gluconate sodium salts were used. In fish and other invertebrates sodium and chloride transport is independent, although often linked (Towle, 1993; Evans et al., 1999). The data presented here support the chloride-independence of sodium influx in *Daphnia magna*.

Comparison with other aquatic organisms

Disturbances in sodium balance with pH have been well documented in both fish and decapod crustaceans (see Vangenechten et al., 1989; Wood, 1989). By contrast, little is known regarding the effect of pH on sodium metabolism in smaller freshwater crustaceans. Potts and Fryer (1979) described inhibited sodium uptake with low pH in two Cladoceran species, while Havas et al. (1984) noted inhibitory effects of acid on sodium metabolism in daphnids exposed to soft water. These latter authors also demonstrated that the sodium influx component of sodium metabolism exhibited greater inhibition in soft than in hard water (Havas et al., 1984), a pattern somewhat contrary to that observed in the present study. The conclusions in this study were somewhat confounded by testing the effect of calcium in natural waters that varied considerably in sodium content (Havas et al., 1984). As sodium uptake is a saturable, facilitated process, the response of the sodium concentration tested will be highly dependent on its relationship to the affinity and capacity of the transport process, stressing the value of the kinetic approach used herein.

It was, nevertheless, somewhat surprising in our study that high calcium, and low pH, eliminated sodium influx in *Daphnia magna*. It has been well known for some time that calcium is an important ameliorator of the physiological perturbations induced by exposure of freshwater fish to acid waters (see Wood, 1989). In laboratory experiments the protective role of increased calcium levels has been explained in terms of a restoration of branchial tight junction integrity, compensating for the initial displacement of junctional calcium by high proton levels (McDonald et al., 1980; McDonald, 1983). The increased calcium thus acts to limit the enhanced sodium efflux component caused by acid water. In freshwater crustaceans, however, it appears that the influx, not the efflux, component is the primary mediator of lowered sodium status. Whole body sodium levels in the current study mirrored trends in sodium influx closely, suggesting little influence of sodium efflux (Fig. 1 cf. Fig. 3). In the freshwater crayfish *Orconectes*, it was noted that sodium efflux was relatively acid-resistant (Wood and Rogano, 1986), supporting the results of an earlier study (Shaw, 1960). Furthermore, Potts and Fryer (1979) failed to delineate any significant effect of calcium on acid-induced

changes in sodium efflux in *Daphnia magna* and *Acantholeberis curvirostris*. It is also of interest to note that increased water hardness appears to offer little protective effect against the toxicity of the sodium antagonist, silver, to *Daphnia magna* (e.g. Bury et al., 2002). Evidence therefore suggests that freshwater crustaceans are fundamentally different from freshwater fish in terms of their physiological response to the modifying influence of calcium on acid-exposed sodium metabolism.

In direct contrast to fish, it is only in hard water that acidification of the medium becomes problematic with regards to sodium influx in *Daphnia magna*. This could be related to calcium metabolism. *Daphnia magna* moult every 2–4 days (Peltier and Weber, 1993). Each moulting event is associated with massive fluxes in mineral status, as the exoskeleton is sloughed and a new exoskeleton is remineralised (Wheatly and Gannon, 1995). Given the relatively low external calcium levels associated with freshwaters and the high frequency of *Daphnia* moulting, it is likely that considerable ion uptake resources are devoted to effective calcium scavenging. The severe inhibition of sodium uptake in the presence of high calcium conditions, as possibly mediated by competitive interactions at a $2Na^+/H^+$ exchanger, could explain why *Daphnia* differ from freshwater fish, and indeed other freshwater crustaceans with longer moulting cycles. Ellis and Morris (1995) ascribed an apparently anomalous ion regulation response to acidification in a freshwater crayfish to perturbation in calcium metabolism, supporting a similar conclusion in *Daphnia*.

Methodological considerations

There are considerable methodological difficulties associated with studying sodium transport mechanisms in *Daphnia magna*. Their small size limits the ability to examine sodium influx in isolated tissues, cell types or membrane surfaces. All of these techniques have been crucial for an understanding of sodium metabolism in fish and euryhaline crustaceans (see Evans et al., 1999; Ahearn et al., 2001).

In the current study sodium influx was examined across the whole animal. Sodium influx thus represents the summed effects of sodium taken up across the epipodite apical surface, that transported across the basolateral surface to the haemolymph, and also sodium that may be absorbed *via* the gastrointestinal pathway. Each of these membrane barriers to transport may handle sodium by distinct mechanisms, resulting in whole body sodium uptake patterns that may differ somewhat from sodium uptake discerned in larger organisms using homogenous preparations. Nevertheless the data presented here suggest that sodium influx in *Daphnia magna* is achieved by sodium–proton exchange, a mechanism that would explain the sensitivity of these organisms to acidified environments and waterborne calcium levels.

This work was supported by a NSERC Collaborative Research and Development Grant Project (CRDPJ 257740-02) with co-funding from Kodak Canada Inc. C.M.W. is supported by the Canada Research Chair program. C.N.G. is supported by a generic office chair.

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