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THE EFFECT OF AMBIENT pH ON ELECTROLYTE REGULATION DURING THE POSTMOULT PERIOD IN FRESHWATER CRAYFISH *PROCAMBARUS CLARKII*

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Summary

The effects of ambient pH on postmoult net fluxes of Ca, acidic/basic equivalents (H^+, NH_4^+/OH^-, HCO_3^-), Na and Cl^-, total body Ca, haemolymph pH and electrolyte status were assessed in the freshwater crayfish *Procambarus clarkii* (Girard). Variables were monitored for 5 days postmoult in acidic (pH5.2; H_2SO_4) or alkaline (pH9.2; KOH) artificial tap water (ATW) and compared with those in control (pH7.4) tap water. In control ATW there was an initial net influx of Ca ($+2700\,\mu mol\,kg^{-1}\,h^{-1}$) and titratable basic equivalents ($+5000\,\mu mol\,kg^{-1}\,h^{-1}$) that declined with time. Calcium uptake accounted for 40% of total body Ca ($0.49\,mmol\,g^{-1}$); haemolymph Ca concentration remained constant. Haemolymph pH was initially relatively alkalotic (7.7) but recovered within 24h. A 20% haemolymph dilution by water uptake at ecdysis necessitated uptake of Cl^- and Na for the first 2–3 days postmoult ($+1000\,\mu mol\,kg^{-1}\,h^{-1}$). In acidic ATW, Ca and basic equivalent uptake were both 60% reduced during the first 3–4 days and total body Ca was reduced by 37%. Chloride and Na uptake and haemolymph [Cl^-] were decreased. In alkaline ATW, Ca and basic equivalent uptake were elevated by 30% for the first 2 days and haemolymph alkalosis was maintained. Sodium and Cl^- balance were unaffected. Thus, ambient pH affects Ca and basic equivalent fluxes associated with postmoult calcification. Regulation of Na and Cl^- levels is also impaired in acidic ATW.

Introduction

Poorly buffered fresh waters are susceptible to pH alteration occurring either naturally or as a result of man's activities. Atmospheric acids are formed when industrial gaseous emissions are precipitated as sulphuric, nitric and hydrochloric acids (Graedel and Crutzen, 1989). Acid rain has become a major environmental problem, especially below pH5.0 (Sprules, 1975). Environmental alkalization occurs when alkaline coal ash wastes rich in calcium oxide are discharged along river banks (Shaw, 1981; Peters *et al.* 1985). In certain athalassohaline lakes (major ionic constituents are $MgSO_4$, Na_2SO_4 and $NaHCO_3/Na_2CO_3$) the pH is naturally around 9.5 (Cooper *et al.* 1987).

The moulting cycle of crustaceans is critical for their survival and growth. Postmoult crustaceans are particularly susceptible to environmental water chemistry because they

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depend heavily on external electrolytes to calcify the new cuticle with calcium carbonate and to restore extracellular electrolyte and acid–base imbalances (Wheatly, 1993). According to Cameron (1985), calcification of the postmoult cuticle occurs *via* the overall reaction:

$$Ca^{2+} + HCO_3^- = CaCO_3 + H^+ . \quad (1)$$

Although modest amounts of Ca are stored between moults in freshwater crustaceans, the majority of the required Ca originates from the external water (Greenaway, 1985; Wheatly, 1990). Since HCO_3^- is required for calcification and protons are produced, the process is directly affected by ambient pH. Postmoult crayfish must also take up Na and Cl^- to correct a haemolymph dilution caused by uptake of fresh water at ecdysis (Wheatly and Ignaszewski, 1990). For detailed reviews of electrolyte regulation in postmoult crustaceans, see Cameron and Wood (1985), Cameron (1985, 1989, 1990), Wheatly and Ignaszewski (1990) and Wheatly (1993).

Freshwater crustacean populations appear to be very sensitive to acidification (Leivestad *et al.* 1976; Havas *et al.* 1984) and alkalization (Shaw, 1981). Crayfish populations have been observed in naturally acid (pH5.8; Huner and Barr, 1991) and alkaline waters (pH10). While certain ecological aspects of pH sensitivity have been addressed in crayfish (France, 1981, 1987*a*,*b;* Berrill *et al.* 1985; Davies, 1989), the underlying physiological responses to extreme pH have only been examined during intermoult (Morgan and McMahon, 1982; Järvenpää *et al.* 1983; McMahon and Stuart, 1985, 1989; Hollett *et al.* 1986; Wood and Rogano, 1986; Mauro and Moore, 1987; Patterson and deFur, 1988). Postmoult crayfish are less resistant to low pH (LC_{50} 3.5; Malley, 1980) than are intermoult crayfish (LC_{50} 2.5; Morgan and McMahon, 1982). When maintained at pH5.0 (Malley, 1980), crayfish typically have poorly calcified exoskeletons as a result of reduced postmoult Ca uptake. There are no existing studies on the physiology of intermoult or postmoult crayfish exposed to basic water. In fish, mortality due to exposure to low and high pH is associated with failure of electrolyte and acid–base regulation (McDonald and Wood, 1981; McDonald, 1983; Wright and Wood, 1985; Wilkie and Wood, 1991).

The goal of the present study was to assess the effect of sublethal acidic (pH5.2) and alkaline (pH9.2) pH exposure (compared with control, pH7.4) on postmoult fluxes of Ca and associated ions, total body Ca and haemolymph pH and electrolyte status in the crayfish *Procambarus clarkii.*

Materials and methods

Maintenance of crayfish

Crayfish, *Procambarus clarkii* (Girard), of both sexes were obtained from Louisiana State University Agricultural Centre. They were kept in groups of 4–8 in 30l aquaria under natural photoperiod at 21˚C and fed three times a week with chopped liver or shrimp pellets (Hartz Mountain Corporation). The water was recycled and aerated through a bottom filter and replaced once a week with dechlorinated thermo-equilibrated tap water. Gainesville tap water has the following electrolyte composition (in $mmol\,l^{-1}$):

Na, 0.55; K, 0.04; Ca, 0.58; Mg, 0.43; Cl^-, 0.73; titratable alkalinity, 1.8 and pH7.8. The crayfish were allowed to acclimate for at least 2 weeks before experiments started (Morgan and McMahon, 1982).

Experimental protocol

The experiments were carried out in artificial tap water (ATW) adapted from Greenaway (1970, 1974*a*,*b*) to resemble the electrolyte concentrations in local tap water. It contained (in $mmol\,l^{-1}$): $NaHCO_3$, 1.18; $KHCO_3$, 0.03; $CaSO_4.2H_2O$, 0.57; $MgCl_2.6H_2O$, 0.52; and control (neutral) ATW had a pH of 7.4–7.6 and a titratable alkalinity of 1.1. For experimental solutions, the pH of ATW was modified by gradual titration with either $1mol\,l^{-1}$ H_2SO_4 to reach pH5.2 (acidic ATW) or $1mol\,l^{-1}$ KOH to reach pH9.2 (alkaline ATW). Water pH and total carbon dioxide content (T_{CO_2}) were measured.

Net electrolyte fluxes with experimental water

Experiments were performed on 30 juvenile postmoult crayfish (1.32±0.10g). Immediately after ecdysis had occurred naturally, animals were placed in up to 500ml (this was adapted according to size) of control, acidic or alkaline ATW (using 10 animals for each treatment). The ATW was continuously aerated and renewed every 24h. Whole-animal net fluxes of Ca, Na, Cl^-, titratable alkalinity and ammonia (NH_3+NH_4^+) with the experimental water were monitored over 24h flux periods for a total of 5 days following ecdysis. A water sample was removed at the start (initial, i) and end (final, f) of each 24 h flux period and kept at 4°C until analyzed. Since no buffer was added to the ATW, pH did vary during the course of the flux period, but only by 0.3–0.5 units.

Total body calcium

In a second experimental series, 28 immediately postmoult crayfish (1.25±0.08g) were exposed to control, acidic or alkaline ATW following the procedure outlined above for a period of 5 days. They were then killed and frozen for subsequent determination of total body calcium.

Haemolymph pH and electrolyte levels

In a third experimental series, postmoult crayfish were sampled for haemolymph pH and electrolytes. Different series of crayfish were used for flux studies and haemolymph sampling because of the known disturbing effects of handling on whole-animal electrolyte fluxes. For this experiment, 24 crayfish of mean mass 12.4±0.5g were used, to avoid haemolymph depletion by repetitive sampling (total haemolymph volume around $0.28ml\,g^{-1}$ bodymass; Wood and Rogano, 1986). The volume of ATW was correspondingly increased to around 1.0–1.8l (depending upon individual animal size) so that the ion uptake mechanisms remained above their saturation concentrations as previously determined (see Wheatly and Ignaszewski, 1990).

On the day of ecdysis (day 0) a prebranchial haemolymph sample was rapidly collected with a 500 μl gas-tight Hamilton syringe from the base of a walking leg. Haemolymph pH was measured immediately after collection (200 μl) and the remaining sample (100 μl)

was frozen for subsequent electrolyte analysis. Crayfish were then exposed to control or experimental pH for 5 days (N=8 in each treatment) and sampled daily.

Analytical procedures and calculations

Water pH was measured using a combination pH electrode and pH meter (Radiometer PHM 84) calibrated with Fisher buffers (pH7.0, 4.0 and 10.0). The water T_{CO_2} was measured using a Capni-Con analyzer (Cameron Instruments Co.) adapted for use with water samples as outlined by Cameron and Wood (1985). Water concentrations of Na and Ca were determined after appropriate dilution (1:40 in 0.1% $CsCl_2$ for Na and 1:20 in 0.1% $LaCl_3$ for Ca) using an atomic absorption spectrophotometer (Perkin Elmer 5000). Chloride concentration was measured by coulometric titration (Radiometer CMT 10) using standards of 1 and 2mmol l^{-1}. Titratable alkalinity was determined by titrating an air-equilibrated 5ml sample with 0.02mol l^{-1} HCl either to pH4.0 (ATW and alkaline ATW) or to pH3.5 (for acidic ATW; McDonald and Wood, 1981). Ammonia was measured using the phenolhypochlorite method of Solorzáno (1969). Net flux of each electrolyte X was calculated in μmol kg^{-1} h^{-1} as:

$$J_X = \frac{([X]_i - [X]_f)V1000}{tW}, \quad (2)$$

where i and f refer to initial and final water concentration (mmol l^{-1}), V is flux volume (l), t is elapsed time (h) and W is mass (kg). By convention, a negative value indicates net electrolyte loss and a positive value net uptake by the animal. The apparent net basic equivalent flux was calculated as the sum of the titratable alkalinity and ammonia fluxes, taking consideration of signs (discussed by McDonald and Wood, 1981). This variable does not distinguish between uptake of basic equivalents and excretion of acidic equivalents or *vice versa*. It is an operational term that includes flux of the following acidic or basic equivalents: H^+/NH_4^+ or OH^-/HCO_3^- (Wood and Rogano, 1986).

For total body calcium measurements, crayfish were thawed and weighed. Samples were then ashed for 12h at 450°C in a muffle furnace (Lindberg model 51894). The ashes were then digested in concentrated HCl, diluted so that Ca concentration could be read on the atomic absorption spectrophotometer, and back-calculated to total body Ca content (mmol g^{-1} wet mass).

Haemolymph pH was measured using an IL pH capillary electrode (20982) attached to an IL 213 blood gas analyzer, calibrated with precision buffers of pH6.840 and 7.384 and thermostatted to 22°C. Haemolymph electrolytes were measured using modified analytical procedures outlined above for water. Dilutions for haemolymph were 1:8000 for Na samples and 1:400 for Ca samples. Chloride concentration was measured on a 5 μl subsample against a standard of 150mmol l^{-1}.

Statistical analyses

Data are expressed as means ± S.E.M. with number of observations in parentheses (N). Effects within (days of exposure) and between (pH) treatments were compared using repeated-measures analysis of variance (ANOVA) (Ott, 1988). Whenever there was a significant interaction between days and pH exposure, a Student–Newman–Keuls' test

(Sokal and Rohlf, 1969) was used for multiple comparisons. Flux measurements were also compared with zero by means of a modified *t*-test enabling a data set to be compared with a single point (Bailey, 1981). For total body calcium content, means were compared between pH treatments using a one-way ANOVA and a multiple comparisons test was performed using a Student-Newman-Keuls' test. Haemolymph electrolytes and pH were analyzed using a repeated-measures ANCOVA (Abacus Concepts, Super Anova). Again, if there was a significant interaction between days and pH exposure, a multiple comparisons test was performed using a Student–Newman–Keuls' test. Statistical significance was accepted at $P<0.05$ throughout.

Results

Control ATW (pH=7.4–7.6) had a T_{CO_2} of 1.13±0.01mmol l^{-1} (N=3). In acidic ATW (pH=5.2), T_{CO_2} decreased to 0.83±0.13mmol l^{-1} (N=3), whereas in alkaline ATW (pH=9.2) T_{CO_2} increased to 1.36±0.08mmol l^{-1} (N=3).

Net electrolyte fluxes with experimental water

Postmoult crayfish in control ATW showed an initial net uptake of Ca (around +2700 $\mu mol kg^{-1} h^{-1}$; Fig. 1A), which decreased steadily with time to around +500 $\mu mol kg^{-1} h^{-1}$ by day 5. This was accompanied by a net uptake (+5000 $\mu mol kg^{-1} h^{-1}$) of basic equivalents (Fig. 1B) that was 80% attributable to net titratable base uptake (Fig. 1C). The remaining 20% originated from net ammonia excretion (Fig. 1D). All three variables (basic equivalents, titratable base and ammonia) decreased linearly over the 5 days postmoult but remained significantly above zero.

In acidic ATW there was a 55–65% reduction in Ca net uptake (averaging +800 $\mu mol kg^{-1} h^{-1}$; Fig. 1A), mainly on the first 3 days postmoult (see statistical summary in Table 1). Net basic equivalent influx was similarly 70% inhibited on the first 4 days postmoult (Fig. 1B). The titratable base uptake was 75% lower for the first 3 days postmoult, with mean values around +1000 $\mu mol kg^{-1} h^{-1}$ (Fig. 1C). Meanwhile, net ammonia efflux was reduced (-550 $\mu mol kg^{-1} h^{-1}$) on the first day (Fig. 1D). Under acid conditions, ammonia and titratable base flux contributed 33% and 67%, respectively, to the total basic equivalent flux.

In alkaline ATW, Ca net influx exhibited a 30% increase on the first 2 days postmoult (averaging +3550 $\mu mol kg^{-1} h^{-1}$; Fig. 1A). Similarly, influxes of net basic equivalents and net titratable base were higher on the first 2 days postmoult (Fig. 1B,C, values averaging +6500 $\mu mol kg^{-1} h^{-1}$) but net basic equivalent uptake was significantly lower than control on day 4. Ammonia net efflux was reduced on day 1 postmoult (Fig. 1D), and the flux measured was not significantly different from zero. In alkaline ATW, ammonia net efflux only contributed 5–10% to the net basic equivalent influx.

In control ATW, postmoult crayfish exhibited net uptake of Cl^- and Na (Fig. 2A,B), initially at rates of +1350 and +900 $\mu mol kg^{-1} h^{-1}$ respectively. Within 3 days for Cl^- and 2 days for Na, influxes had dropped to rates that were not significantly different from zero, suggesting that ion balance had been re-established. In acidic ATW, the pattern of net Cl^- uptake over 5 days was not significantly different from the control pattern

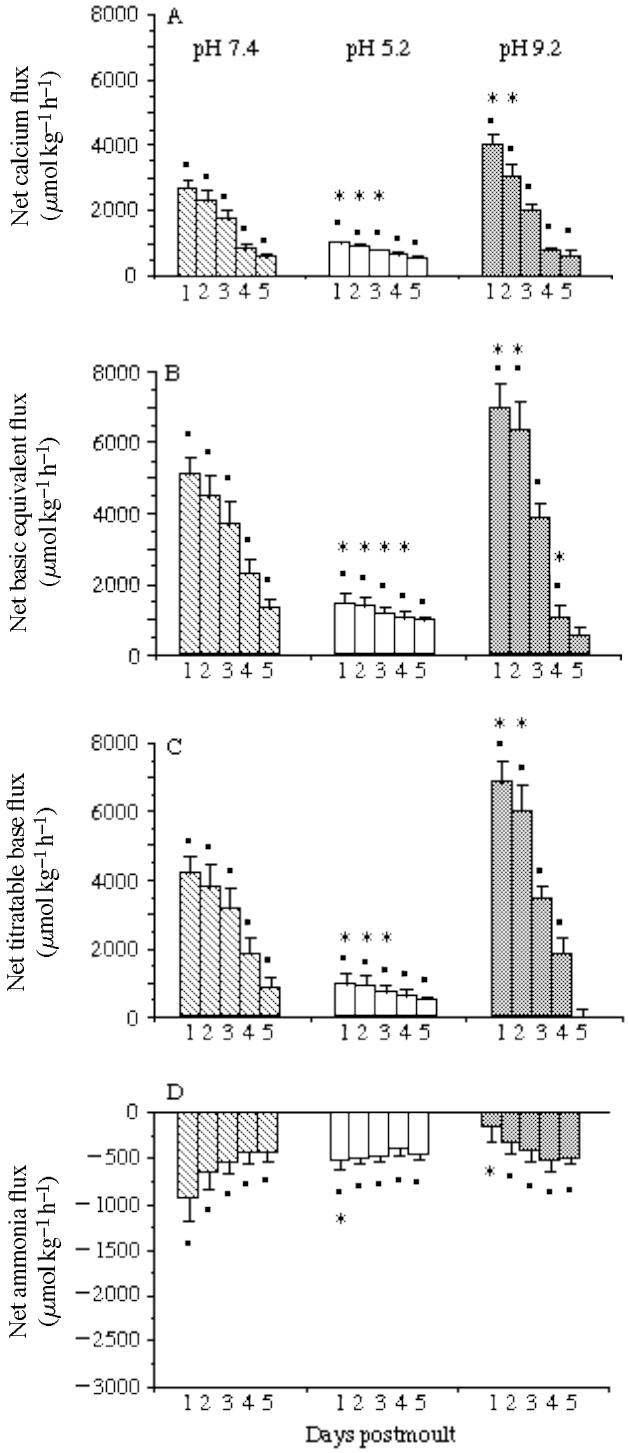
A
pH 7.4
pH 5.2
pH 9.2
Net calcium flux
(μmol kg⁻¹ h⁻¹)
8000
6000
4000
2000
0
B
Net basic equivalent flux
(μmol kg⁻¹ h⁻¹)
C
Net titratable base flux
(μmol kg⁻¹ h⁻¹)
D
Net ammonia flux
(μmol kg⁻¹ h⁻¹)
−500
−1000
−1500
−2000
−2500
−3000
1 2 3 4 5
Days postmoult

Fig. 1

Fig. 1. Whole-animal net flux of (A) calcium, (B) basic equivalents, (C) titratable base and (D) ammonia for the first 5 days postmoult in *Procambarus clarkii* (mean mass 1.32±0.10g) at 21°C. Bars represent mean values ± S.E.M. for three different pH treatments. Control ATW had a pH of 7.2–7.6 (N=10; mean mass 1.46±0.25g); experimental treatments were either pH5.0–5.2 (acidic ATW; N=10; mean mass 1.36±0.08g) or pH9.0–9.2 (alkaline ATW; N=10; mean mass 1.10±0.12g). Asterisks denote differences from the control value on the appropriate day (repeated-measures ANOVA, P<0.05). Dots represent values that are significantly different from zero. By convention, positive values indicate net uptake and negative values net output by the animal.

(Fig. 2A), and no significant differences were found between days. Net uptake of Na, however, was not significantly different from zero on any of the 5 days postmoult (Fig. 2B), unlike controls, which showed significant net uptake for the first 2 days postmoult. Net uptake of Cl^- was significantly higher than the control on day 1 in alkaline ATW (Fig. 2A). Alkaline pH had a significant effect on Na net uptake, which was reduced compared to the control (Fig. 2B). Starting on the second day, the net Na efflux was not significantly different from zero, whereas controls showed significant net uptake for 2 days.

The total flux of each electrolyte with the experimental water was summed over the entire 5 days of measurement (Table 2).

Total body calcium

After 5 days in control ATW, total body Ca was 0.49mmol g^{-1} wetmass (Table 3). In acidic ATW, total body Ca was significantly lower than control values (0.31mmol g^{-1} wetmass), whereas it was unchanged in alkaline ATW.

Haemolymph pH and electrolyte levels

Crayfish in control ATW had a haemolymph pH of 7.7 at ecdysis (Fig. 3A) that declined to 7.5 after 24h and remained at that level. An identical trend was observed upon transfer to acidic ATW (Fig. 3A; statistical summary in Table 4). On day 1, crayfish exposed to alkaline ATW (Fig. 3A) had a haemolymph pH of 7.75, which was significantly higher than control values. Haemolymph pH remained at that level for the next 2 days. On day 4, pH declined to 7.6, which was still significantly elevated compared with control values. By day 5, haemolymph pH was similar to control values.

Following ecdysis, crayfish in control ATW had a haemolymph Ca level of 9.6mmol l^{-1}. Haemolymph Ca concentration did not change significantly over 5 days postmoult in crayfish in control, acidic or alkaline ATW (Fig. 3B). Following ecdysis, haemolymph Cl^- concentration was initially reduced to 128mmol l^{-1} (Fig. 3C) but subsequently recovered to 157mmol l^{-1} on day 1, reaching values around 168mmol l^{-1} after 5 days. Crayfish exposed to acidic ATW (Fig. 3C) had Cl^- levels of 116mmol l^{-1} at ecdysis. After 1 day, Cl^- levels had increased to 151mmol l^{-1}, exhibiting the same trend as in control animals. On days 4 and 5, Cl^- levels exhibited a secondary drop compared with the control. Exposure to alkaline ATW (Fig. 3C) had no effect on postmoult Cl^- concentration. Immediately following ecdysis, Na concentration was 122mmol l^{-1}

Table 1. *Summary of statistical analysis (repeated-measures ANOVA) for electrolyte net fluxes with external water under three different pH treatments*

Flux	Source of variation	d.f.	*F*-value	*P*-value
J_{Ca}	Between subjects			
	pH	2	18.1	0.0001
	Within subjects			
	Days	4	110	0.0001
	Days × pH	8	20.7	0.0001
J_B	Between subjects			
	pH	2	23.0	0.0001
	Within subjects			
	Days	4	84.4	0.0001
	Days × pH	8	22.0	0.0001
J_{TB}	Between subjects			
	pH	2	17.0	0.0001
	Within subjects			
	Days	4	73.7	0.0001
	Days × pH	8	22.9	0.0001
J_{Amm}	Between subjects			
	pH	2	0.99	0.38
	Within subjects			
	Days	4	0.55	0.70
	Days × pH	8	6.4	0.0001
J_{Cl}	Between subjects			
	pH	2	1.52	0.24
	Within subjects			
	Days	4	81.1	0.0001
	Days × pH	8	2.49	0.016
J_{Na}	Between subjects			
	pH	2	4.04	0.03
	Within subjects			
	Days	4	5.65	0.0004
	Days × pH	8	1.57	0.14

d.f., degrees of freedom; J_{Ca}, calcium net flux; J_B, basic equivalent net flux (calculated); J_{TB}, titratable base net flux; J_{Amm}, ammonia net flux; J_{Cl}, chloride net flux; J_{Na}, sodium net flux.

(Fig. 3D); this increased to 137mmol l^{-1} on day 1, with values levelling off around 146mmol l^{-1} on days 2–5. Exposure to acidic and alkaline ATW (Fig. 3D) had no effect on this trend.

Discussion

Control (neutral) ATW

Postmoult mineralization in *Procambarus clarkii* in neutral ATW was characterized by a net uptake of Ca and basic equivalents (Fig. 1A,B) agreeing with previous studies

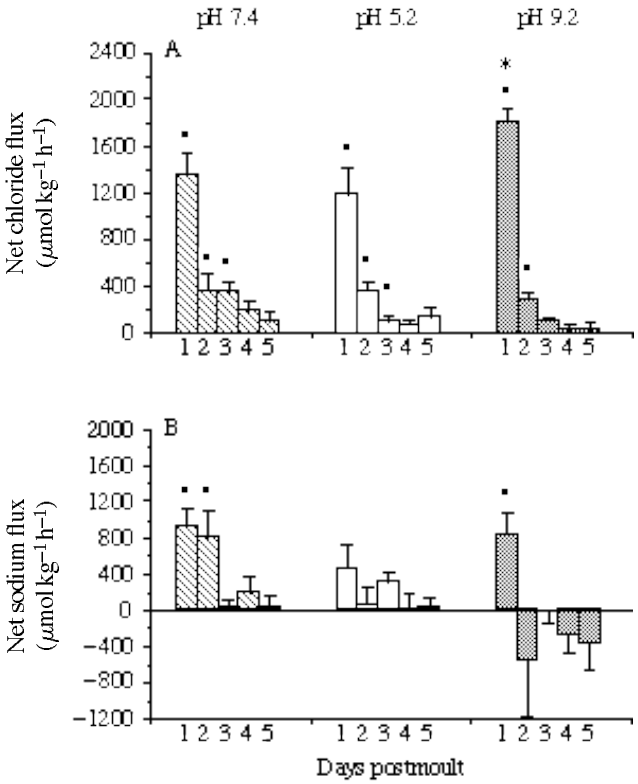

Fig. 2. Whole-animal net flux of (A) chloride and (B) sodium for the first 5 days postmoult in *Procambarus clarkii*. For additional details, consult legend to Fig. 1.

Table 2. *Total electrolyte flux (mmolkg^{-1}) with the experimental water over 5 days in postmoult crayfish under three different pH treatments*

Treatment	J_{Ca}	J_B	J_{TB}	J_{Amm}	J_{Na}	J_{Cl}
Control ATW (pH7.4–7.6)	193.8±23.8	403.4±49.5	331.6±55.2	−71.7±17.3	47.9±9.9	56.7±5.7
Acidic ATW (pH5.0–5.2)	92.2±3.8	143.8±19.4	87.5±19.4	−56.3±6.8	21.7±10.1	44.6±4.3
Alkaline ATW (pH9.0–9.2)	250.7±22.1	449.6±26.7	403.2±37.5	−46.4±12.0	−8.8±19.9	53.8±5.1

By convention, positive values correspond to uptake and negative values correspond to output by the animal (N=10); values are mean ± S.E.M.

Symbols are explained in Table 1; ATW, artificial tap water.

(Wheatly and Ignaszewski, 1990). Comparable Ca net influxes have been reported for the crayfish *Austropotamobius pallipes* (Greenaway, 1974*b*) and *Orconectes virilis* (Malley, 1980). In sea water, blue crabs with net Ca and basic equivalent influxes as high as +6000 μmolkg^{-1} h^{-1} and +12500 μmolkg^{-1} h^{-1}, respectively, have been reported

Table 3. *Total body calcium content (mmol g^{-1}) and total whole-animal Ca influx (mmol g^{-1} from Table 2) after 5 days postmoult at three different pH treatments*

Treatment	Total body calcium	Total calcium flux
Control ATW (pH7.4–7.6)	0.49±0.01 (12)	0.19±0.02 (10)
Acidic ATW (pH5.0–5.2)	0.31±0.02 (8)*	0.09±0.004 (10)
Alkaline ATW (pH9.0–9.2)	0.49±0.02 (8)	0.25±0.02 (10)

The asterisk denotes significant difference from control (ANOVA; P=0.0001).
ATW, artificial tap water.
Values are mean ± S.E.M. (N).

(Cameron, 1985; Cameron and Wood, 1985), possibly relating to increased availability of Ca and HCO_3^- in sea water as opposed to fresh water. In both cases, the stoichiometry between Ca and basic equivalent uptake rates is 1:2, as one might predict from the calcification equation. Greenaway (1974*b*) similarly reported that Ca influx rate dropped over time, although it remained significant for several weeks in 10g crayfish. A decrease in ammonia excretion with time (Fig. 1D) has been observed in other studies (Mangum *et al.* 1985*a*; Wheatly and Ignaszewski, 1990). Muscle atrophy during late premoult is believed to facilitate exuviation from constricting regions of the old exoskeleton (Mangum *et al.* 1985*a*) and may account for elevated ammonia excretion in the early postmoult period. Alternatively, it may be indirectly related to the exchange of counterions such as sodium that are taken up in the postmoult period (see below).

Postmoult crayfish also exhibited significant net uptake of Cl^- and Na (Fig. 2A,B) for 2–3 days to correct the haemolymph dilution created by uptake of fresh water at ecdysis. Net Cl^- influx was greater in magnitude, which agrees with another study (Wheatly and Ignaszewski, 1990). Unidirectional flux analysis using radiotracers (Wood and Rogano, 1986; Wheatly, 1989) has demonstrated that the crayfish integument has a greater permeability to Cl^- than to Na.

Allometric relationships derived for total body Ca of intermoult *Austropotamobius* (Greenaway, 1985) and *Procambarus clarkii* (Wheatly, 1990) predict values of 0.452 and 0.491mmol g^{-1}, respectively, for a 1.25g crayfish. Perfect correspondence between these and the values measured in day 5 postmoult crayfish (Table 3) suggests that calcification was virtually completed. The net Ca influx integrated over 5 days postmoult in a different group of similarly sized crayfish (Table 3, values taken from Table 2) suggests that about

Fig. 3. Changes in haemolymph pH (A), calcium (B), chloride (C) and sodium (D) as a function of days postmoult under three different pH treatments. Control treatment represents pH7.4 exposure and experimental treatments were pH5.2 or pH9.2. Day 0 refers to ecdysis, when haemolymph was sampled in control pH prior to transfer of the crayfish to experimental pH. Bars represent mean values ± S.E.M., N=8. Asterisks indicate significant differences from control for corresponding days.

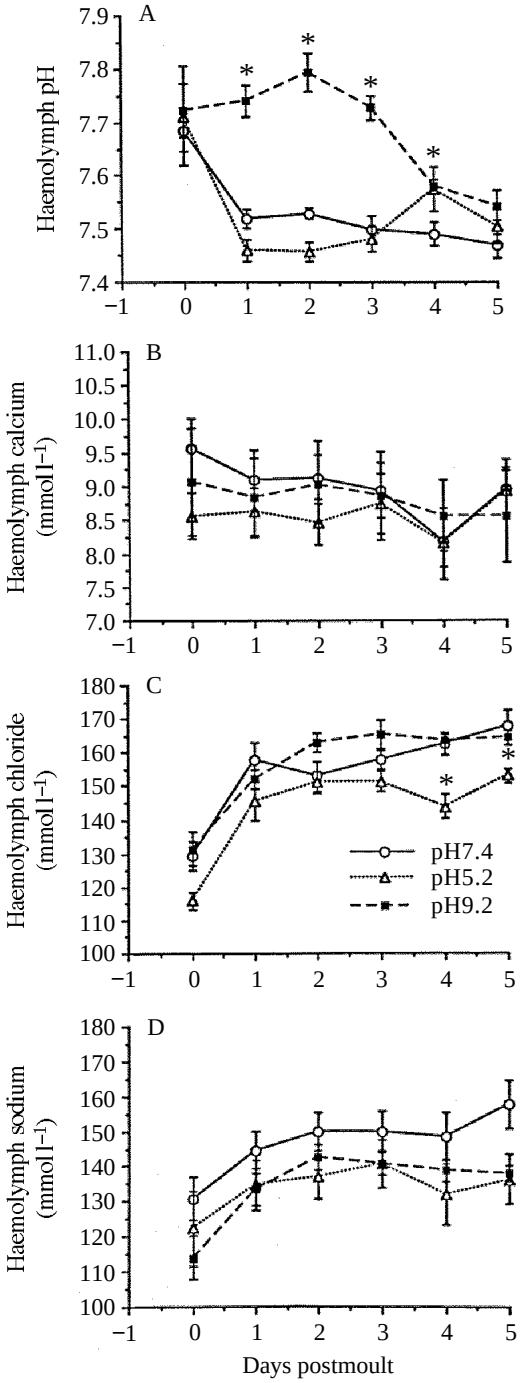
A
Haemolymph pH
7.9
7.8
7.7
7.6
7.5
7.4
B
Haemolymph calcium (mmol l⁻¹)
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
C
Haemolymph chloride (mmol l⁻¹)
180
170
160
150
140
130
120
110
100
pH7.4
pH5.2
pH9.2
D
Haemolymph sodium (mmol l⁻¹)
−1
0
1
2
3
4
5
Days postmoult

Fig. 3

Table 4. *Summary of statistical analysis (repeated-measures ANCOVA) for haemolymph electrolytes under three different pH treatments*

Electrolyte	Source of variation	d.f.	*F*-value	*P*-value
pH	Covariate			
	Day 0	1	3.26	0.09
	Within subjects			
	Days × pH	8	12.0	0.0001
Calcium	Covariate			
	Day 0	1	6.19	0.02
	Within subjects			
	Days × pH	8	0.54	0.83
Chloride	Covariate			
	Day 0	1	8.8	0.008
	Within subjects			
	Days × pH	8	2.18	0.04
Sodium	Covariate			
	Day 0	1	27.5	0.0001
	Within subjects			
	Days × pH	8	0.67	0.72

Day 0 refers to the day of ecdysis.
The interaction factor was used as the significance level.
The symbols are explained in Table 1.

40% of total body Ca originated from the external medium. The remaining 60% was presumably stored in various body tissues (Greenaway, 1985).

Intermoult extracellular pH of *Procambarus* at 22˚C is typically 7.5 (Wheatly and Henry, 1992). The alkalosis observed at ecdysis (Fig. 3A) is probably associated with elevated haemolymph [HCO_3^-] resulting from premoult exoskeletal $CaCO_3$ reabsorption as well as HCO_3^- influx from the medium. However, since haemolymph [HCO_3^-] was not measured in the present study, one can only speculate that the alkalosis was of metabolic not respiratory origin. The alkalosis was compensated within 24h, agreeing with previous studies on crayfish (Dejours and Beekenkamp, 1978) and blue crabs (Mangum *et al.* 1985*a*,*b*; deFur *et al.* 1988).

In this study, the haemolymph data are not strictly comparable with the flux data since they were obtained from a separate series of crayfish that were an order of magnitude greater in mass. Separate studies in our laboratory on the allometry of postmoult calcification in neutral ATW (Wheatly *et al.* 1991) concluded that diffusional and active flux rates are both greater in smaller crayfish, commensurate with an increased surface area to volume ratio. Haemolymph data for crayfish ranging in mass from 3 to 30 g (Wheatly, 1993; M. G. Wheatly, unpublished observations; and the present study), however, showed no differences between circulating electrolyte levels in either intermoult crayfish or immediately after ecdysis, with the exception of Cl^-. Postmoult Cl^- values were initially lower in larger crayfish, suggesting that the values presently

reported for 12g crayfish may have overestimated the changes expected in the group used for flux experiments.

The present study agrees with Wheatly (1993) that haemolymph [Ca] remains constant during the postmoult period (Fig. 3B) and at essentially intermoult levels (around 10mmol l^{-1}, Morgan and McMahon, 1982), suggesting a delicate balance between Ca uptake and deposition into the new exoskeleton. The fact that extracellular Ca concentration remained constant in the face of dilution of other major circulating electrolytes suggests that total circulating Ca actually increased around ecdysis. Greenaway (1974*a*) confirmed that total extracellular Ca was elevated in postmoult *Austropotamobius*, primarily in the bound moiety; ionized calcium remained constant. Intermoult Cl^- and Na levels in *Procambarus* are typically around 190mmol l^{-1} (Morgan and McMahon, 1982; Wheatly, 1993). A 30% drop in both upon ecdysis (Fig. 3C,D) indicates a substantial haemodilution. Restoration of ion balance coincided with significant influx from the external medium (Fig. 2).

Acidic ATW

The fact that Ca and basic equivalent net uptake were both similarly reduced in acidic ATW (Fig. 1A,B) indicates that their uptake mechanisms are linked, as previously suggested (Cameron, 1985; Cameron and Wood, 1985; Wheatly and Ignaszewski, 1990). Malley (1980) similarly showed in postmoult *Orconectes virilis* that Ca influx was impaired in acid water and completely inhibited below pH4.0. Low pH combined with high aluminium concentration reduced Ca uptake further (Malley and Chang, 1985). Cameron (1985) demonstrated that Ca and basic equivalent uptake in postmoult blue crabs were both virtually eliminated in acid sea water. This effect could not be reproduced in high-CO_2 sea water (which lowered pH by a similar amount), leading him to the conclusion that the response in acidified sea water was due to the lowering of ambient T_{CO_2} rather than an effect of pH *per se*. In the present study, ambient T_{CO_2} was approximately halved by acid titration, as was basic equivalent influx rate. In a related study (Zanotto and Wheatly, 1990) we repeated these measurements in decarbonated water and found that Ca and basic equivalent uptake were reduced in neutral decarbonated ATW compared with uptake in neutral non-decarbonated ATW. Flux rates were the same in neutral and acidic decarbonated ATW, confirming Cameron's (1985) conclusions. Other studies have demonstrated that removing external HCO_3^- lowers Ca uptake in postmoult crayfish by 60% (Greenaway, 1974*b*) and virtually eliminates the basic equivalent uptake (M. G. Wheatly and A. T. Gannon, unpublished observations). The fact that Ca uptake decreases in all these studies suggests that the Ca uptake mechanism is HCO_3^--dependent.

Total body Ca was one-third reduced after 5 days in acidic ATW compared with control values (Table 3). Crayfish naturally exposed to acidified lakes have a decreased exoskeletal calcium content (Malley, 1980; Appelberg, 1985; France, 1987*a*). In either case this could result from reduced postmoult Ca uptake (as demonstrated above) and/or erosion of existing exoskeletal $CaCO_3$ under acid conditions. Extracellular acidosis has been shown to induce exoskeletal dissolution in intermoult crustaceans (see review by

Wheatly and Henry, 1992). In a separate experiment we observed a sizeable Ca efflux from isolated shed exuviae into acidic ATW (F. P. Zanotto and M. G. Wheatly, unpublished observations). Direct erosion of $CaCO_3$ at the carapace/water interface could oppose branchial uptake, contributing to reduced net influx under acid conditions.

In acidic ATW, postmoult crayfish exhibited an identical haemolymph pH and Ca profile to those in neutral ATW (Fig. 3A,B). Two studies on acid exposure (H_2SO_4; pH4.0) of intermoult crayfish (*Procambarus clarkii*, Morgan and McMahon, 1982; *Orconectes propinquus*, Wood and Rogano, 1986) report acid–base and ion responses that are exactly counter to those observed in postmoult. A progressive metabolic acidosis, originating from H^+ entry at the gills, was accompanied by an elevation in circulating [Ca] and net Ca efflux (Wood and Rogano, 1986), both originating from exoskeletal erosion. McMahon and Stuart (1989) reported that the carapace Ca content decreased significantly after 21 days of acid exposure.

The pronounced reduction in net basic equivalent uptake in acidic ATW (Fig. 1B) primarily reflected changes in net titratable base uptake (Fig. 1C). In addition, on the first day postmoult there was a significant reduction in ammonia excretion (Fig. 1D). Recent reviews indicate that ammonia excretion in aquatic animals can occur by ionic or non-ionic diffusion as well as by Na^+/NH_4^+ exchange and NH_4Cl+NaCl cotransport (Kormanik and Cameron, 1981; Evans and Cameron, 1986). Based on the analysis of Wright and Wood (1985), acid exposure should raise the diffusional gradients for both NH_3 and NH_4^+, thereby increasing ammonia excretion. Since a reduction in Na influx accompanied the decrease in ammonia excretion (Fig. 2B), Na^+/NH_4^+ exchange may have been inhibited. In acid-exposed intermoult crayfish, ammonia excretion is either unaffected (Wood and Rogano, 1986) or increases (Mauro and Moore, 1987).

Haemolymph Cl^- levels were significantly reduced on days 4 and 5 (Fig. 3C), when whole-animal uptake had essentially ceased. This would suggest that extracellular Cl^- was being sequestered in a body fluid compartment other than the haemolymph. Haemolymph pH increased on the same days that [Cl^-] dropped, implicating the operation of a Cl^-/HCO_3^- exchanger. Acid-exposed intermoult crayfish similarly showed a reduction in circulating [Cl^-] (Appelberg, 1985; Wood and Rogano, 1986), although in this case it was attributed to net efflux due to reduced unidirectional influx. Other studies (Morgan and McMahon, 1982; Hollett *et al.* 1986) report no effect of acid exposure on haemolymph [Cl^-] in intermoult crayfish.

Net sodium uptake was reduced in acid ATW (Fig. 2B and Table 2). Shaw (1960) reported that low external pH reduced unidirectional Na influx in intermoult crayfish while passive efflux remained unchanged. A net efflux of Na was observed in acid-exposed intermoult crayfish (Wood and Rogano, 1986) caused initially by a reduction in the unidirectional influx (competition between H^+ and Na for a common carrier), followed by an increase in diffusive efflux. Recent experiments (M. G. Wheatly and A. T. Gannon, unpublished observations) indicate that postmoult Na uptake may be linked to Ca uptake, since removal of external Na reduces Ca uptake by 50%.

In the present study, Na fluxes were not significantly different from zero during acid exposure, explaining why haemolymph Na levels remained constant (Fig. 3D) and agreeing with studies on intermoult *Procambarus* (Morgan and McMahon, 1982) and

Cambarus robustus (Hollett *et al.* 1986). In acid-exposed *Orconectes* (Wood and Rogano, 1986), however, a net Na efflux was correlated with reduced circulating levels.

The differences between physiological responses to acute acid in postmoult and intermoult crayfish may be attributable to the degree of acidification (generally lower in intermoult studies, pH 4 *versus* pH5.2) as well as to differences in water hardness and the species under study. In fish, ambient Ca concentration affects acid toxicity (McDonald, 1983). In hard water, the major physiological effect is a metabolic acidosis that originates from Na loss in excess of Cl^-; in soft water, H^+ influx does not occur but equimolar losses of Na and Cl^- result in rapid mortality due essentially to ion failure. Havas *et al.* (1984) similarly showed that low pH had more debilitating effects on Na regulation in *Daphnia* in soft water than in hard water. The present study was conducted in water of intermediate hardness (Ca=0.5mmol l^{-1}), as was the study by Morgan and McMahon (1982; 1.1mmol l^{-1}), which may explain why the electrolyte disturbances were less pronounced. Studies conducted by Malley (1980) and Wood and Rogano (1986), however, were both in soft water (0.7 and 0.1mmol l^{-1} respectively).

Laboratory and field studies indicate that certain crayfish genera, such as *Procambarus* and *Cambarus*, are far more resistant to low pH than other genera, such as *Orconectes* (Berrill *et al.* 1985; Hollett *et al.* 1986; Davies, 1989; McMahon and Stuart, 1989) because of their evolutionary history. The genus *Procambarus* is commonly found in swampy areas and therefore may have acquired resistance to acid conditions. The evolution of orconectoid and cambaroid lines from a *Procambarus*-like ancestor has been under different conditions of water chemistry. *Cambarus* originated in mountain streams under soft water conditions and so may have become preadapted to withstanding low pH stress. Differing acid sensitivity may also be related to the seasonal timing of reproductive and moulting events.

Recent research (McMahon and Stuart, 1989) has shown that the associated anion is also an important factor in the physiological responses to acid exposure. Both acute and chronic (7–60 days) exposure to nitric acid in *Procambarus* produce less pronounced disturbances in ion/acid–base status than does H_2SO_4. The initial haemolymph acidosis is less severe and more rapidly compensated, and exoskeletal $CaCO_3$ mobilization does not occur.

Alkaline ATW

The increased basic equivalent uptake observed in alkaline ATW (Fig. 1B) may be attributed to higher ambient T_{CO_2}. Cameron (1985) showed that increased ambient HCO_3^- in sea water caused an increase in HCO_3^- influx into postmoult blue crabs, although a corresponding change in Ca influx was not observed. In the present study, net Ca uptake increased initially in proportion to the titratable base uptake (Table 2 and Fig. 1A), suggesting again that the uptake mechanisms are linked and possibly that the increase in Ca uptake is secondary to the HCO_3^- uptake. Over the entire 5-day period, total Ca uptake was only 30% increased over control levels (Table 2), which was insufficient to be reflected in the total body Ca content (Table 3) or haemolymph [Ca] (Fig. 3B).

There was a significant decrease in net ammonia efflux on day 1 postmoult (Fig. 1D). A corresponding reduction in net Na influx was not observed in this case (Fig. 2B). Since high external pH would increase the external P_{NH_3} and reduce external [NH_4^+] (Wright and Wood, 1985), the observed reduction in ammonia excretion could be interpreted as evidence that there is an NH_3 diffusional component to the net ammonia excretion, at least during the initial postmoult period when the cuticle is soft. Subsequently (day 2–5), ammonia excretion recovered to control levels (Fig. 1D). Without resolving the net Na flux (Fig. 2B) into its unidirectional components, it is difficult to say whether the ammonia excretion occurring at this time was *via* Na^+/NH_4^+ exchange or NH_4^+ diffusion. However, recent experiments (M. G. Wheatly and A. T. Gannon, unpublished observations) indicate that postmoult ammonia excretion persists in Na^+-free medium, suggesting the predominant role of diffusional routes in ammonia excretion. Collectively, the acid and alkaline experiments suggest that ammonia excretion in the postmoult crayfish occurs by a combination of Na^+/NH_4^+ exchange and diffusion of NH_3 or NH_4^+.

Sodium and Cl^- regulation were generally unaffected by exposure to alkaline water. Net Cl^- uptake was initially elevated (Fig. 2A) and both Cl^- and Na balances were re-established a day earlier than in control conditions (Fig. 2A,B). The latter effect may be related to the increased rate of calcification (Fig. 1). However, when summed over the 5-day postmoult period (Table 2), net fluxes of Na and Cl^- were similar to controls. Likewise, circulating [Cl^-] and [Na] showed similar postmoult trends to those control crayfish (Fig. 3C,D). Even in fish, plasma ion disturbances are less pronounced in alkaline water than in acid water.

The haemolymph alkalosis following ecdysis persisted for several days postmoult in alkaline ATW, whereas it had recovered within 24h in control and acid-exposed crayfish (Fig. 3A). Without measuring additional indices of acid–base status, one can only speculate on the origin of this. The maintained alkalosis may be attributed to increased basic equivalent uptake from water (Fig. 1C). Cameron (1985) reported that haemolymph pH increased in postmoult blue crabs experiencing increased basic equivalent uptake in high-HCO_3^- water.

To conclude, this study has outlined the effect of acidic or alkaline exposure on electrolyte regulation during the postmoult period in freshwater crayfish. By affecting the ability to calcify, environmental pH can potentially affect crustacean population dynamics in the wild. To elucidate how electrolyte-transporting mechanisms are affected by ambient pH, it will be necessary to measure unidirectional ion fluxes, which is the focus of our continued studies. The uptake mechanisms for Na and Cl^- in freshwater crustaceans have been reviewed by Ehrenfeld (1974) and Mantel and Farmer (1983). The uptake mechanism for Ca has been modelled for freshwater teleost fish (Fenwick, 1978; Flik *et al.* 1983), although there have been some preliminary studies using crustacean models (Roer, 1980).

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