Physiological Responses to Acid Stress in Crayfish (*Orconectes*): Haemolymph Ions, Acid–Base Status, and Exchanges with the Environment

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Exposure of *Orconectes propinquus* for 5 d to pH = 4.0 (H_2SO_4) in decarbonated soft water ($[Ca^{2+}]$ = 0.20 mequiv·L^{-1}) caused a severe metabolic acidosis and a moderate depression of $[Na^+]$ and $[Cl^-]$ in the haemolymph. Lactate did not accumulate. Acidosis was caused by a large uptake of acidic equivalents from the environmental water, of which more than 95% was stored outside the extracellular compartment after 5 d. Carapace buffering was probably involved, because haemolymph $[Ca^{2+}]$ rose substantially and Ca^{2+} was lost to the environment. Similar net effluxes of K^+ indicated that acidic equivalents also penetrated the intracellular compartment. SO_4^{2-} was also lost during acid exposure. Haemolymph $[Na^+]$ fell more than $[Cl^-]$ because of greater net losses to the water. Unidirectional flux analyses with radiotracers demonstrated that negative net Na^+ and Cl^- balance resulted from partial inhibition of influx components; effluxes were little affected. All flux effects were reversed during 5 d of recovery at pH = 7.5. Haemolymph ionic responses in *Orconectes rusticus* differed in showing a smaller, equimolar reduction of $[Na^+]$ and $[Cl^-]$ and a much larger elevation of $[Ca^{2+}]$. At a mechanistic level, the responses of crayfish to acid stress appear very different from those of teleost fish.

L'exposition pendant 5 jours d'*Orconectes propinquus* à une eau douce décarbonatée ($[Ca^{2+}]$ = 0,20 méquiv·L^{-1}) de pH (H_2SO_4) de 4,0 s'est traduite par une acidose métabolique sévère accompagnée d'une baisse modérée de la teneur en ions Na^+ et Cl^- de l'hémolymphe. Il n'y a pas eu accumulation de lactate. L'acidose résultait d'une importante entrée d'équivalents acides à partir de l'eau du milieu et l'on retrouvait plus de 95 % de ceux-ci emmagasinés à l'extérieur du compartiment extracellulaire après 5 jours. La carapace exerçait probablement un effet tampon, car la teneur en Ca^{2+} de l'hémolymphe s'est élevée de façon appréciable et il y a eu perte de Ca^{2+} dans le milieu. Des pertes nettes semblables de K^+ indiquaient que des équivalents acides avaient aussi pénétré dans le compartiment intracellulaire. Du SO_4^{2-} a aussi été perdu au cours de l'exposition au milieu acide. La teneur en Na^+ de l'hémolymphe s'est abaissée plus que celle en Cl^- suite à des pertes nettes dans l'eau plus importantes. Des analyses par radiotraceurs du transport unidirectionnel vers l'eau ont permis de démontrer que l'équilibre net négatif du Na^+ et du Cl^- résultait d'une inhibition partielle des composantes d'entrée, celles de sortie étant peu modifiées. Tous ces effets sur le transport ont été inversés au cours d'une période de récupération de 5 jours à un pH de 7,5. Les réponses ioniques de l'hémolymphe étaient différentes chez *Orconectes rusticus* en ce que l'on notait une réduction équimolaire des teneurs en Na^+ et en Cl^- moins importante et une augmentation beaucoup plus importante de la teneur en Ca^{2+}. Les mécanismes de réponse de l'écrevisse au stress acide semblent différer de beaucoup de ceux notés chez les poissons téléostéens.

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The physiology of acid stress in teleost fish has been intensively studied, and it is now clear that the gill is the primary site of toxic action (cf. Wood and McDonald 1982; McDonald 1983a; Howells 1984 for recent reviews). Effects on branchial O_2 uptake, acid–base balance, and ionoregulatory mechanisms have been described, with the latter predominating as the cause of toxicity at environmentally realistic levels of acid stress (i.e. pH's $\geq$ 4.0). Crustacean communities are severely affected by environmental acidification (e.g. Leivestad et al. 1976; Singer 1982; France 1983) but physiological responses have received scant attention. Crayfish, however, are a notable exception; effects on Na^+ uptake (Shaw 1960b), postmoult calcification (Malley 1980), haemolymph oxygenation (Järvenpää et al. 1983), and haemolymph ionic and acid–base status (Morgan and McMahon 1982; McMahon and Morgan 1983) have been described. Only one study was done in soft (i.e. low $[Ca^{2+}]$) water (Malley 1980). In the wild, acidification is exclusively a softwater problem, and the fish literature indicates that $[Ca^{2+}]$, the major component of water hardness, has a critical modifying effect on both acid toxicity and the nature of the toxic syndrome (McDonald et al. 1980; McDonald 1983a, 1983b).

Our goal was to assess the physiology of acid stress in crayfish under softwater conditions resembling those in affected regions of eastern Canada where stream pH's may fall as low as 4.0 during snowmelt or rainstorms (e.g. Jeffries et al. 1979; Harvey and Lee 1982). To facilitate comparisons with recent studies on fish under the same conditions (McDonald et al. 1980; Wood and McDonald 1982; McDonald et al. 1983; McDonald 1983b; Höbe et al. 1984), similar techniques for evaluating branchial function and internal regulation have been employed. These include repetitive sampling of haemolymph

for ionic, acid–base, and lactate status, the latter as an index of O_2 transport disturbance, and measurement of the unidirectional exchanges of Na^+ and Cl^- and the net fluxes of acidic equivalents and other ions between the animal and its environment. We chose the crayfish *Orconectes propinquus* because the species is endemic to the acid-threatened region of eastern Canada (Berrill 1978); we used *Orconectes rusticus*, which has been widely introduced throughout the same area, for supplementary measurements because of its larger size.

Materials and Methods

Experimental Animals and Water

Experiments were performed on 130 adult intermoult *O. propinquus* (4.2 ± 0.9 g; $\bar{X}$ ± 1 SE) collected by dipnet from Spencer's Creek, Dundas, Ontario, and 12 intermoult *O. rusticus* (17.1 ± 1.8 g) purchased from Boreal Laboratories, Mississauga, Ontario. Identification was performed by the keys of Crocker and Barr (1968). The animals used did not moult for at least 3 wk prior to and 2 wk following the experiments. For both species, the origins were hard water, as animals of soft water origin were unavailable in sufficient numbers. The composition of the hard water was as follows: $[Ca^{2+}] \cong 1.8$; $[Na^+] \cong 0.6$; $[Cl^-] \cong 0.8$; $[Mg^{2+}] \cong 0.3$; $[K^+] \cong 0.05$; $[SO_4^{2-}] \cong 0.5$; titration alkalinity ≅ 2.0 mequiv·L^{-1}; pH ≅ 8.0. In the laboratory, the animals were first adjusted to experimental temperature (10 ± 1°C) in hard water for 1 wk and then transferred to vigorously aerated artificial soft water for a further 2–3 wk. The loading rate was 0.5 g·L^{-1}, and the water was changed weekly. The animals were fed commercial trout pellets and furnished with pieces of black ABX pipe as refugia to minimize cannibalism. The soft water was prepared as a 1:10 dilution of hard water with distilled water, decarbonated by acidification to pH = 2.8 with H_2SO_4, aerated for 24 h, and then titrated with KOH and NaOH back to the appropriate pH (control = 7.5; acid = 4.0). The composition was as follows: $[Ca^{2+}] \cong 0.20$; $[Na^+] \cong 0.20$; $[Cl^-] \cong 0.15$; $[Mg^{2+}] \cong 0.03$; $[K^+] \cong 0.20$; $[SO_4^{2-}] \cong 0.25$; titration alkalinity ≅ 0.20 mequiv·L^{-1}. One week prior to experimentation, the required crayfish (6–12) were transferred to a smaller tank containing 60 L of freshly made soft water and ABX pipe refugia and thereafter starved to minimize any influence of feeding history on the experimental results. Two days prior to experimentation, each crayfish received a numbered tag. In those animals intended for arterial haemolymph sampling, a small hole was drilled through the carapace directly above the pericardial sinus and sealed with several layers of dental dam and cyanoacrylate glue so as to create a sampling port.

The small size of *O. propinquus* limited the amount of haemolymph which could be withdrawn. Total haemolymph volume is ~0.28 mL·g body weight^{-1} (Kerley and Pritchard 1967). For this reason, only the largest specimens (5–10 g) were used in the haemolymph sampling experiments, and an identically sampled set of control animals were run at neutral pH in all series. The larger *O. rusticus* (14–20 g) were employed in an experiment (series iii) requiring the daily withdrawal of a much larger sample (200 μL) for ionic measurements. All samples were drawn quickly (10–20 s) and with minimal disturbance into ice-cold gas-tight Hamilton syringes while the animal was underwater in its experimental tank. The sampling needle was fitted with a cuff permitting only 2-mm penetration to prevent damage to internal organs.

Experimental Series

(i) Haemolymph acid–base status was assessed on a control day followed immediately by 5 experimental days (repetitive sampling) in *O. propinquus* either kept at pH = 7.5 (n = 9) or exposed to pH = 4.0 (n = 11). A final measurement was taken on day 12. On day 0, haemolymph samples (50 μL) were drawn from the animals in their acclimation/starvation tanks at pH = 7.5; the crayfish were then transferred to identical tanks at either pH = 7.5 or 4.0 for subsequent sampling on days 1–5 and 12. The water was changed on days 5 and 10. Water pH was checked twice daily and adjusted as necessary by addition of 1 *M* KOH or 0.5 *M* H_2SO_4; fluctuations were less than 0.1 pH unit and the total elevation of $[SO_4^{2-}]$ over 5 d was ~0.10 mequiv·L^{-1}.

(ii) Haemolymph ionic status was assessed in *O. propinquus* subjected to similar experimental protocols (pH = 7.5, n = 12; pH = 4.0, n = 16) but sampled only on day 0 (200 μL) and day 5 (200 μL). On day 5, an additional 200 μL was drawn for lactate analysis in five to seven animals from both groups, as well as from a previously unsampled control group.

(iii) In order to follow the temporal development of ionic disturbances, the larger *O. rusticus* were subjected to the same regimes (pH = 7.5, n = 6; pH = 4.0, n = 6) but sampled daily (200 μL) from day 0 through to day 5.

(iv) Unidirectional fluxes of Na^+ and Cl^- and net fluxes of Na^+, Cl^-, K^+, Ca^{2+}, SO_4^{2-} (in five or six animals of each group only), ammonia, titratable acidity, and acidic equivalents were measured on a daily basis in *O. propinquus* on day 0, followed by 5 experimental days either at pH = 7.5 (n = 26) or pH = 4.0 (n = 23). In 10 animals from each group, fluxes were also measured during 5 d of recovery at pH = 7.5. The schedule was arranged so that the day 1 flux represented the first 5 h of exposure to low pH, and the day 1 flux during recovery represented the first 5 h of return to pH = 7.5. The animals were held in the 60-L tanks at appropriate pH as in the previous series, except during the actual 5-h flux measurements. For these, the animals were placed individually in small, well-aerated plastic containers filled with water of the correct pH. The control series served as a check on the possible disturbing effects of the brief handling and air exposure (<5 s) involved in these transfers. The ratio of water to animals was 50:1. These conditions represented the best compromise between water pH control and analytical sensitivity on the basis of 10 preliminary experiments employing various times and volume ratios. Water pH typically rose by about 0.3 unit (at pH = 4.0) over the 5-h run. All water used in the flux experiments was taken from two freshly made batches, either pH = 7.5 or pH = 4.0; both were spiked with 0.185 kBq (5×10^{-3} μCi) $^{22}Na \cdot mL^{-1}$ and 0.092 kBq (2.5×10^{-3} μCi) $^{36}Cl \cdot mL^{-1}$ (NEN). Fluxes were calculated from changes of concentration of substances in the closed system over the 5-h period.

Analyses and Calculations

Haemolymph acid–base status was assessed by standard theory and Radiometer electrode methodology as described previously (McDonald et al. 1979; Wood and Randall 1981). The measurements were difficult because of the low sample volumes (50 μL) and the very rapid clotting (<30 s) which necessitated disassembly and cleaning of the pH electrode after

every measurement, and precluded direct P_{CO_2} measurements. Approximately 25 μL was used for direct pHa measurement in a Radiometer E5021 capillary electrode at 10°C, and exactly 20 μL was injected into a miniature Cameron (1979) chamber (volume = 1.1 mL) for measurement of total CO_2 (Ca_{CO_2}). Pa_{CO_2} (in torr; 1 torr = 133.32 Pa) and $[HCO_3^-]$ (incorporating CO_3^{2-} and carbamino-CO_2) were calculated indirectly via the Henderson–Hasselbalch equation as outlined by McDonald et al. (1979) using pK' and αCO_2 values from the nomograms of Truchot (1976) at the appropriate temperature and ionic strength.

The concentration of acidic equivalents (ΔH_h^+) added to the haemolymph over time ("haemolymph metabolic acid load") was calculated as

$$(1)\quad \Delta H^+_h = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta\,(pH_1 - pH_2)$$

where β is the nonbicarbonate buffer capacity of the haemolymph in slykes (i.e. $-\Delta HCO_3^- \cdot \Delta pH^{-1}$; Wood and Randall 1981). In vitro tonometry of pooled, declotted haemolymph from three and four specimens of *O. propinquus*, using techniques described by McDonald et al. (1979), yielded values of 7.4 and 8.5 slykes, respectively. Wilkes et al. (1980) reported β values for 10 individual *O. rusticus* in the range 7–11 slykes. A β value of 8 slykes was therefore used in the present calculations.

Haemolymph (200 μL) was immediately fixed in 400 μL of ice-cold 8% $HClO_3$ for subsequent lactate determination by the lactic dehydrogenase/NADH method (Sigma 1977). We did not observe the end-point drift reported in this assay by Graham et al. (1983), so chelating agents were not employed. Haemolymph for other ions was stored at −20°C. Upon thawing, the sample was mechanically disrupted and centrifuged at 10 000 × *g* for 5 min to separate clots. $[Na^+]$, $[K^+]$ (Eel MkII), and $[Ca^{2+}]$ (Coleman 20) were detected by flame photometry, appropriate swamping being used to remove interference effects. $[Cl^-]$ was determined by coulometric titration (Radiometer CMT10).

Water from the flux experiments was analyzed immediately for titratable alkalinity by titration of air-equilibrated 10-mL samples to pH = 4.00 with 0.02 *N* HCl as described by McDonald and Wood (1981). The remainder of the sample was frozen for later determination of Na^+, K^+, and Ca^{2+} (as for haemolymph); Cl^- (by coulometric titration on a Buchler-Cotlove 4-2000 chloridometer); total ammonia (by a micromodification of the phenolhypochlorite method of Solorzano 1969); and in some cases SO_4^{2-} (by the turbidometric method of Jackson and McCandless 1978). Net flux rates of each substance were calculated as

$$(2)\quad J_{net} = \frac{([X]_i - [X]_f)\cdot V}{t \cdot W}$$

where *i* and *f* refer to initial and final concentrations (nanoequivalents per millilitre), *V* the volume of the system (millilitres), *t* the elapsed time (hours), and *W* the body weight (grams). Thus, net losses by the animal have a negative sign, and net gains a positive sign. By reversing the *i* and *f* terms, the net titratable acidity flux was calculated from the titratable alkalinities. The sum of the titratable acidity (J_{net}^{TA}) and ammonia (J_{net}^{Amm}) fluxes, signs considered, yielded the net flux of acidic equivalents ($J_{net}^{H^+}$) which derives from the original principles outlined by Maetz (1973). As McDonald and Wood (1981) pointed out, this method does not distinguish between ammonia movement in the NH_3 and NH_4^+ forms, nor between the net excretion of acidic equivalents and the net uptake of basic equivalents, or vice versa. Fortunately this does not matter in terms of net acid–base balance.

Since ^{36}Cl is a pure β-emitter, while ^{22}Na is a mixed γ- and β-emitter, the cpm of each in a sample for unidirectional flux determinations could be separated by difference after counting in a γ-counter and a scintillation counter, as described by Wood et al. (1984). Unidirectional influxes (J_{in}) of Na^+ and Cl^- were calculated as outlined by Maetz (1956):

$$(3)\quad J_{in} = \frac{(R_i - R_f)\cdot V}{SA \cdot t \cdot W}$$

where R_i and R_f are initial and final radioactivities (cpm per millilitre), SA the mean specific activity (cpm per nanoequivalent) over the flux period, and the other symbols as in eq. (2). Preliminary analysis by the logarithmic model of Kirschner (1970) gave virtually identical results. In those instances where calculated internal specific activity exceeded 5% of external specific activity, backflux correction was performed as described by Maetz (1956). Unidirectional effluxes (J_{out}) were calculated by the conservation equation

$$(4)\quad J_{out} = J_{net} - J_{in}.$$

Data have been expressed as means ± SE (n) unless otherwise stated. The significance ($p \leq 0.05$) of differences between means was assessed using Student's two-tailed *t*-test, using either a paired (within groups) or unpaired (between groups) design as appropriate. Differences ($p \leq 0.05$) in mortality between control and experimental groups were assessed by a χ^2-test, one-tailed.

Results

Exposure of *O. propinquus* to pH = 4.0 in soft water for 5 d resulted in 28% mortality (19/69), significantly higher than the control mortality of 11% (6/53). At least two instances of the latter were due to cannibalism. In those animals in which the acid exposure was continued for 12 d, mortality rose to 54% (7/13), significantly higher than 10% (1/10) in the control group. There was no mortality in *O. rusticus* during 5 d at pH = 4.0.

Haemolymph Acid–Base and Ionic Status

Acid exposure caused a progressive fall in haemolymph pHa in *O. propinquus* from ~7.8 to ~7.3 by days 4 and 5 (Fig. 1A). While the acidosis was accompanied by a rise in Pa_{CO_2} during the first 2 d (Fig. 1C), the major effect was a progressive decline in haemolymph $[HCO_3^-]$ from ~7 to ~2 mequiv·L^{-1} (Fig. 1B). At 12 d, pHa was the same as at 5 d, but $[HCO_3^-]$ had fallen to less than 1 mequiv·L^{-1}, while Pa_{CO_2} (~0.7 torr) had dropped to about 25% of the day 0 level. Sampling caused small but significant changes in all three factors in the control group (Fig. 1), but these contributed marginally, if at all, to the effects seen in the experimental group. On a pH–HCO_3^- diagram (Fig. 2A), it was clear that the predominant effect until the end of day 4 was a metabolic acidosis with HCO_3^- loss at more or less constant Pa_{CO_2}. Thereafter, the compensation which stabilized pHa on days 5–12 was respiratory, i.e. a very large reduction in Pa_{CO_2}. The calculated "metabolic acid load" (ΔH_h^+) in the haemolymph was almost 4 mequiv·L^{-1} after only 24 h of acid exposure, reached

TABLE 1. Concentrations of major electrolytes (mequiv·L^{-1}, means ± 1 SE (n)) in arterial haemolymph of *O. propinquus* before (day 0) and after (day 5) exposure to either pH = 4.0 (experimental) or pH = 7.5 (control) for 5 d. *Significantly different ($p < 0.05$) from corresponding day 0 value.

	Day 0		Day 5	
	Control	Experimental	Control	Experimental
Na^+	191.9±9.2 (11)	192.9±8.3 (11)	186.7±6.5 (11)	147.2±11.2 (11)*
Cl^-	182.7±10.2 (12)	177.9±12.1 (11)	175.6±10.4 (12)	145.7±15.3 (11)*
K^+	3.65±0.62 (11)	3.45±0.34 (16)	3.22±0.49 (11)*	2.86±0.27 (16)*
Ca^{2+}	20.40±1.53 (12)	19.95±1.71 (16)	22.91±2.06 (12)	27.28±1.41 (16)*

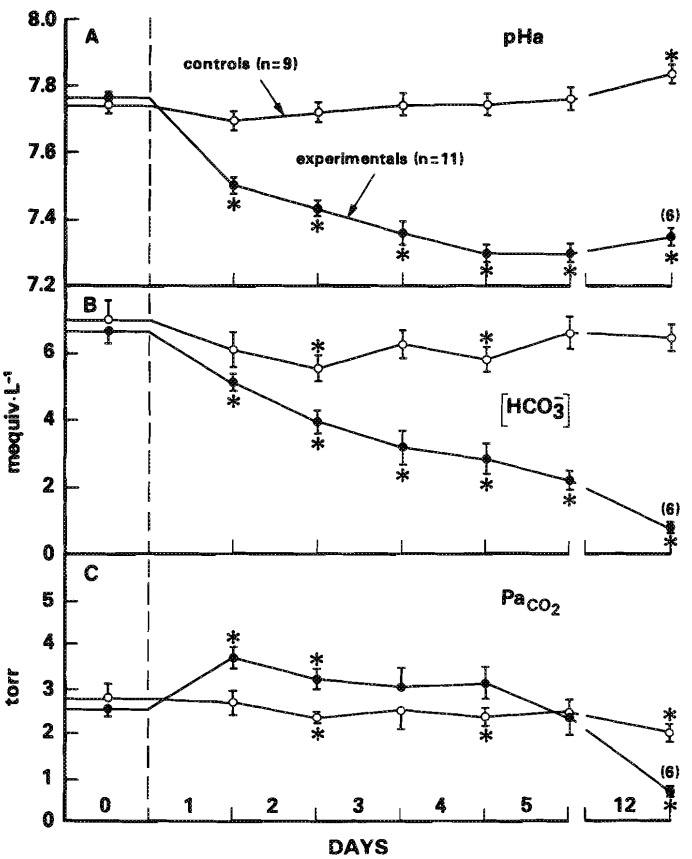

FIG. 1. Changes in (A) pH, (B) bicarbonate concentration (incorporating CO_3^{2-} and carbamino-CO_2), and (C) the partial pressure of CO_2 in the arterial haemolymph of *O. propinquus* during exposure to pH = 4.0 (1 torr = 133.332 Pa). The experimental animals were transferred from pH = 7.5 to pH = 4.0 after day 0. The control animals were kept at pH = 7.5 throughout. Data are means ± 1 SE. Asterisks indicate points significantly different from day 0 value for each group.

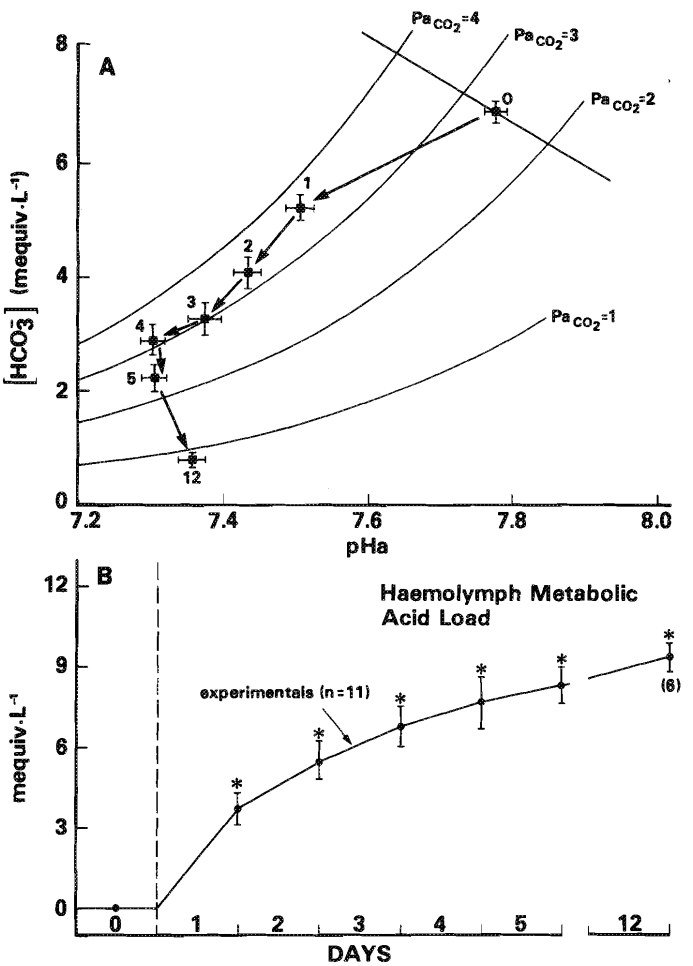

FIG. 2. (A) Changes in the acid–base status of arterial haemolymph in *O. propinquus* during 12 d of exposure to pH = 4.0, displayed on a pH–HCO_3^- diagram. HCO_3^- incorporates CO_3^{2-} and carbamino-CO_2. The Pa_{CO_2} isopleths are in torr (1 torr = 133.332 Pa). Data are means ± 1 SE; n as in part B. The diagonal line plotted through the day 0 value is a typical nonbicarbonate buffer line for haemolymph with a slope (β) of 8 slykes. (B) Calculated haemolymph metabolic acid load (ΔH_n^+) in *O. propinquus* during 12 d of exposure to pH = 4.0. Data are means ± 1 SE. Asterisks indicate points significantly different from the day 0 value which by definition is 0.

8 mequiv·L^{-1} after 5 d, and ~9.5 mequiv·L^{-1} by 12 d. This accumulation of acidic equivalents was not associated with a buildup of lactate, which remained very low in the haemolymph in both control (0.89 ± 0.10(7) mequiv·L^{-1}) and experimental animals (0.57 ± 0.16(5) mequiv·L^{-1}) after 5 d of exposure (Table 1) versus 0.68 ± 0.19(6) mequiv·L^{-1} in previously unsampled control animals. None of these values differed significantly ($p > 0.05$).

Environmental acidity also affected haemolymph ionic status in *O. propinquus* (Table 1). After 5 d, haemolymph [Na^+] and [Cl^-] had both fallen significantly; the decline in [Na^+] (~46 mequiv·L^{-1}) was significantly greater than that in Cl^- (~32 mequiv·L^{-1}). These ions did not change in the control animals kept at pH = 7.5. [K^+], which was present in much lower concentration in the blood, also fell significantly but this is of doubtful importance, as similar changes occurred

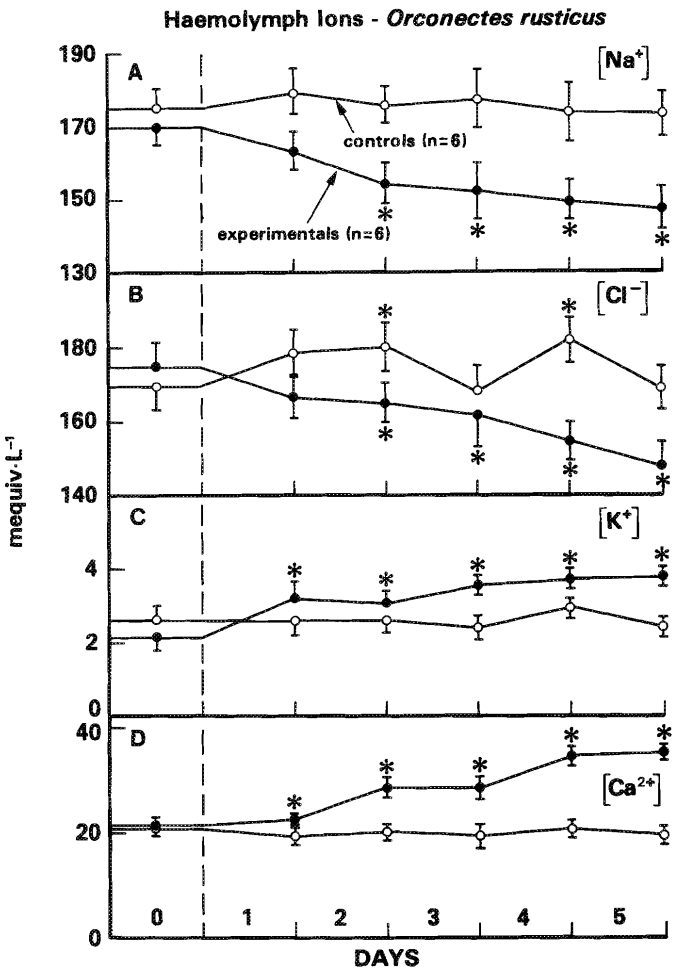

FIG. 3. Changes in (A) Na^+, (B) Cl^-, (C) K^+, and (D) Ca^{2+} concentrations in the arterial haemolymph of *O. rusticus* during exposure to pH = 4.0. The experimental animals were transferred from pH = 7.5 to pH = 4.0 after day 0. The control animals were kept at pH = 7.5 throughout. Data are means ± 1 SE. Asterisks indicate points significantly different from day 0 value for each group.

in the controls. On the other hand, haemolymph $[Ca^{2+}]$ increased significantly, while there was no change in the controls.

Haemolymph ions were measured on a daily basis in *O. rusticus* in an attempt to discern temporal patterns (Fig. 3A). However, the response appeared somewhat different than in *O. propinquus* because $[Na^+]$ and $[Cl^-]$ fell progressively to a much lesser extent and by equivalent amounts (~25 mequiv·L^{-1}) over 5 d. Furthermore, haemolymph $[K^+]$, which had decreased in *O. propinquus* (Table 1), almost doubled in *O. rusticus* (Fig. 3C). $[Ca^{2+}]$ rose progressively (Fig. 3D); the overall increase (~15 mequiv·L^{-1}) was about twice as large as that in *O. propinquus* (~7 mequiv·L^{-1}). Ion levels in the controls were relatively stable, the only apparent complication due to sampling being a small elevation of haemolymph $[Cl^-]$ on days 2 and 4 (Fig. 3B).

Ionic and Acidic Equivalent Exchanges with the Environment

The flux experiments were performed to see whether ionic and acid–base exchanges with the environmental water explained the observed haemolymph changes in *O. propinquus*. For the sake of simplicity, the data from those crayfish which were followed for 5 d of recovery after acid exposure have been pooled with those where the experiments were terminated after 5 d of exposure. In both control and experimental groups, there were relatively slight differences in the data between the two treatments, and none of these were significant on either day 0 or day 5. Furthermore, for all fluxes with the exception of K^+

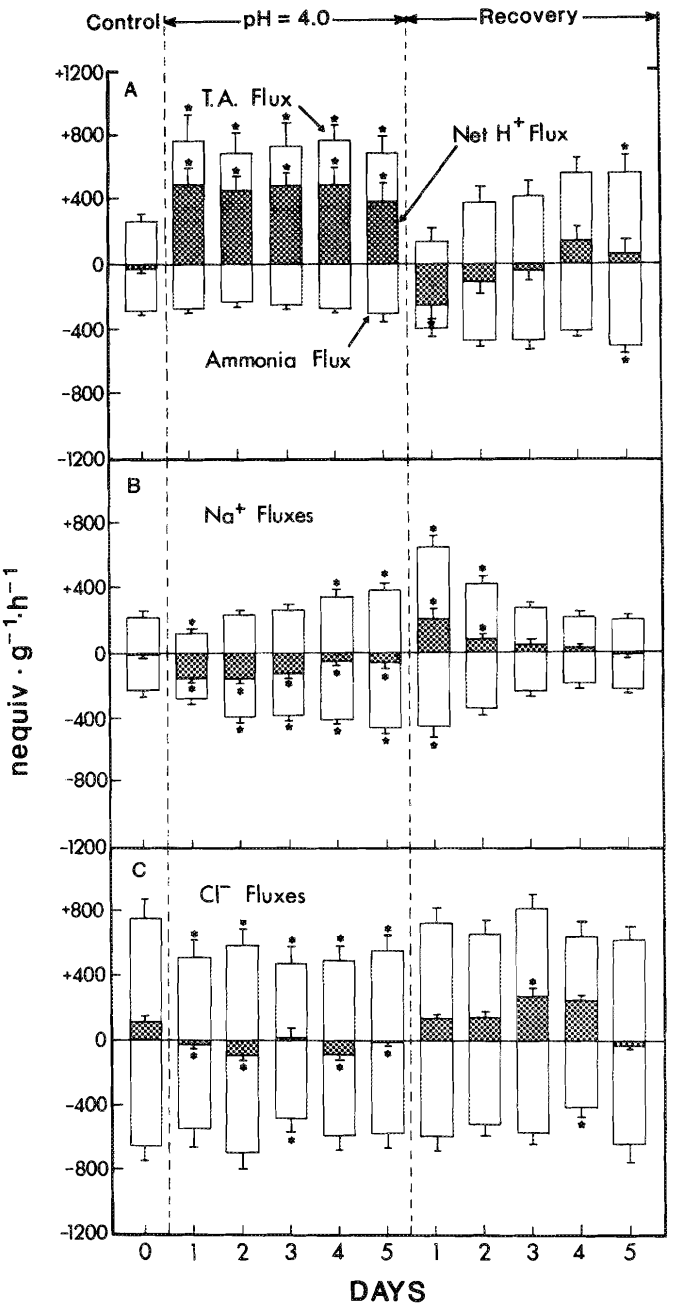

FIG. 4. Flux components in *O. propinquus* under control conditions at pH = 7.5, during 5 d of exposure to pH = 4.0, and during 5 d of recovery at pH = 7.5. (A) TA flux = titratable acidity flux (J_{net}^{TA}, upward bars), ammonia flux (J_{net}^{Amm}, downward bars), and the arithmetic sum of the two, the net acidic equivalent flux ($J_{net}^{H^+}$, stippled bars). (B) Unidirectional Na^+ influx ($J_{in}^{Na^+}$, upward bars), efflux ($J_{out}^{Na^+}$, downward bars), and net flux ($J_{net}^{Na^+}$, stippled bars). (C) Unidirectional Cl^- influx ($J_{in}^{Cl^-}$, upward bars), efflux ($J_{out}^{Cl^-}$, downward bars), and net flux ($J_{net}^{Cl^-}$, stippled bars). Data are means ± 1 SE; n = 23 on days 0–5 and n = 10 on days 1–5 recovery. Asterisks indicate points significantly different from the day 0 value.

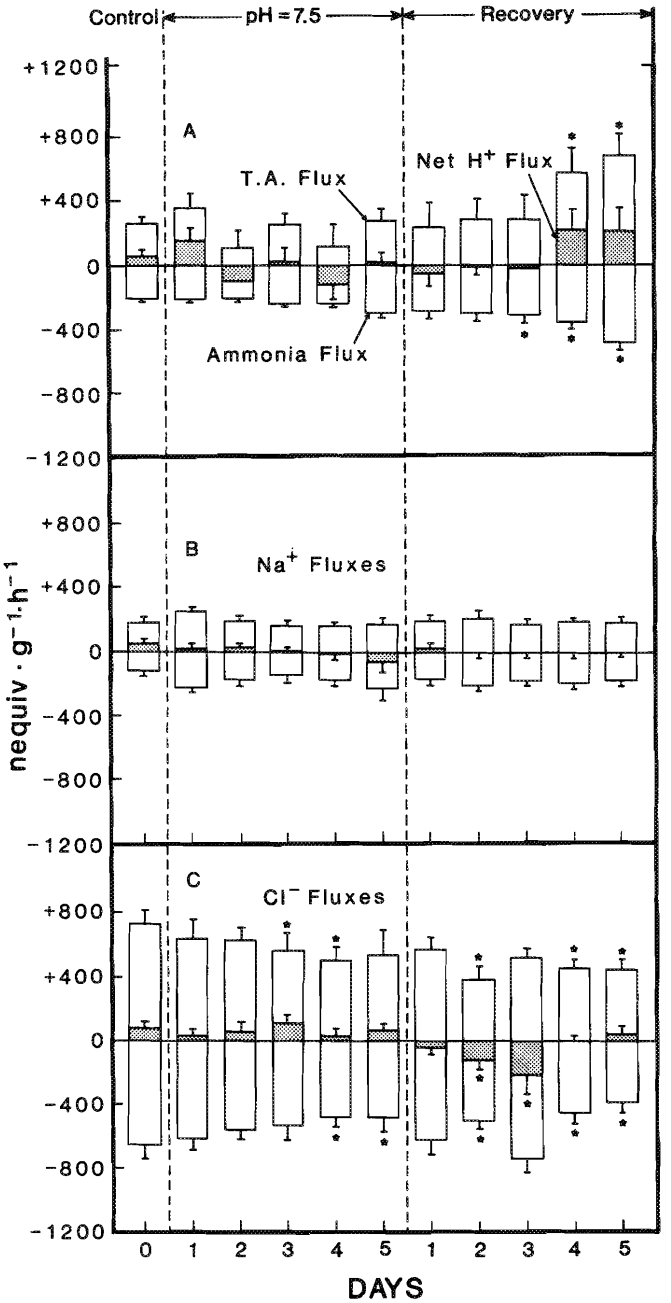

FIG. 5. Flux components in *O. propinquus* under maintained control conditions at pH = 7.5 in a regime otherwise duplicating the experimental regime of Fig. 4; $n = 26$ on days 0–5 and $n = 10$ on days 1–5 recovery. Other details as in Fig. 4.

(Fig. 6A versus 7A), the day 0 values were the same in the control and experimental crayfish.

On day 0 at pH = 7.5, crayfish were in net acid–base balance with the environment, the total ammonia excretion ($J_{net}^{Amm} \cong -250$ nequiv·g^{-1}·h^{-1}) just balancing the titratable acidity uptake ($J_{net}^{TA} \cong +250$ nequiv·g^{-1}·h^{-1}), so that $J_{net}^{H^+}$ was not significantly different from zero (Fig. 4A). The control experiment at pH = 7.5 (Fig. 5A) demonstrated that these exchanges were relatively stable over time except for increases in J_{net}^{TA} and J_{net}^{Amm} during the final 2–3 d of recovery. During 5 d at pH = 4.0, J_{net}^{Amm} remained unchanged, while J_{net}^{TA} increased two- to three-fold, resulting in highly positive values of $J_{net}^{H^+}$ (~ +450 nequiv·g^{-1}·h^{-1}) throughout the exposure period (Fig. 4A). Upon return to pH = 7.5, there was an immediate drop in $J_{net}^{H^+}$ to below the day 0 level. J_{net}^{Amm} was again unchanged, so $J_{net}^{H^+}$ now became significantly negative. On subsequent days of recovery, the fluxes returned to day 0 levels. Therefore the large load of acidic equivalents taken up during 5 d of acid exposure did not appear to be fully excreted during recovery. However, the data must be interpreted with caution, as the actual flux measurements covered only 5 h of each 24-h period. The increases in both J_{net}^{TA} and J_{net}^{Amm} on the last day of recovery (Fig. 4A) can probably be related to the similar effects seen in the control group (Fig. 5A).

Unidirectional influxes and effluxes of Na^+ were both ~200 nequiv·g^{-1}·h^{-1}, resulting in negligible $J_{net}^{Na^+}$ under control conditions (Fig. 4B). The fluxes remained entirely stable during the 11-d control experiment at pH = 7.5 (Fig. 5B). Acid exposure caused an immediate net loss of Na^+ (~ −160 nequiv·g^{-1}·h^{-1}) due to a 50% inhibition of $J_{in}^{Na^+}$ at unchanged $J_{out}^{Na^+}$ (Fig. 4B). However, by day 2, $J_{in}^{Na^+}$ had recovered, and by day 4 and 5 had increased above the control level. In contrast, $J_{out}^{Na^+}$ gradually increased with time. The net effect was a moderation of Na^+ loss after day 2, but $J_{net}^{Na^+}$ still remained significantly depressed on day 5. Immediately upon return to pH = 7.5, $J_{in}^{Na^+}$ increased further to approximately threefold the original day 0 level. As $J_{out}^{Na^+}$ remained unchanged, $J_{net}^{Na^+}$ became highly positive (~ +210 nequiv·g^{-1}·h^{-1}). During the ensuing 4 d of recovery, influx, efflux, and net flux components all gradually returned to their original levels. The total Na^+ losses during 5 d of acid exposure were approximately regained during 5 d of recovery.

At pH = 7.5, unidirectional fluxes of Cl^- (~700 nequiv·g^{-1}·h^{-1}) were about threefold greater than those of Na^+, and a slightly positive net balance occurred (~ +80 nequiv·g^{-1}·h^{-1}; Fig. 4C). The control experiment revealed two complicating features of the protocol (Fig. 5C). Firstly, both $J_{in}^{Cl^-}$ and $J_{out}^{Cl^-}$ slowly declined by approximately 40% over the 11-d experiment, an effect which first became significant on day 3. Secondly, the slightly positive $J_{net}^{Cl^-}$ which persisted through the first 5 d became significantly negative by days 7 and 8, i.e. the second and third days of the recovery period. Despite these complications, it is clear that exposure to pH = 4.0 significantly reduced $J_{net}^{Cl^-}$, generally to negative values, while return to pH = 7.5 permitted full recovery of $J_{net}^{Cl^-}$ (Fig. 4C). On balance, it appeared that more Cl^- was gained during 5 d of recovery than was lost during 5 d of acid exposure. By comparison with the control experiment, these effects on $J_{net}^{Cl^-}$ appeared mainly due to a moderate (~30%) inhibition of $J_{in}^{Cl^-}$ during pH = 4.0 exposure and a stimulation of $J_{in}^{Cl^-}$ upon return to pH = 7.5, with minimal changes occurring in $J_{out}^{Cl^-}$.

On day 0, $J_{net}^{K^+}$ was positive (~ +80 nequiv·g^{-1}·h^{-1}) in the experimental group (Fig. 6A), but approximately zero in the control animals (Fig. 7A); the reason for this difference is unknown. Nevertheless, the control experiment demonstrated stability of K^+ balance over the 11-d period (Fig. 7A). In contrast, $J_{net}^{K^+}$ became significantly negative (~ −80 nequiv·g^{-1}·h^{-1}) during 5 d at pH = 4.0 in the experimental group, an effect which persisted during the first 5 h of recovery at pH = 7.5 (Fig. 6A). By day 2, $J_{net}^{K^+}$ had returned to the day 0 level, but the overall K^+ loss during acid exposure was not completely restored during 5 d of recovery.

Rather surprisingly, Ca^{2+} balance was only moderately affected by environmental acidity. $J_{net}^{Ca^{2+}}$, which was close to zero

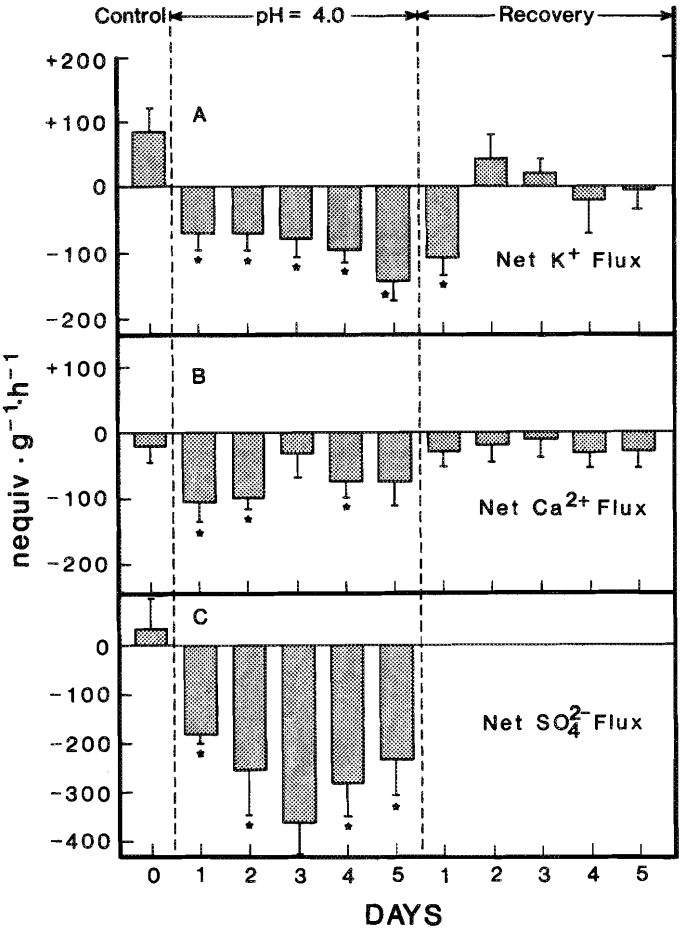

FIG. 6. Net fluxes of (A) K^+, (B) Ca^{2+}, and (C) SO_4^{2-} in *O. propinquus* under control conditions at pH = 7.5, during 5 d of exposure to pH = 4.0, and during 5 d of recovery at pH = 7.5. Data are means ± 1 SE; n = 23 on days 0–5 for K^+ and Ca^{2+}, n = 6 for SO_4^{2-}, and n = 10 on days 1–5 recovery. Asterisks indicate points significantly different from the day 0 values.

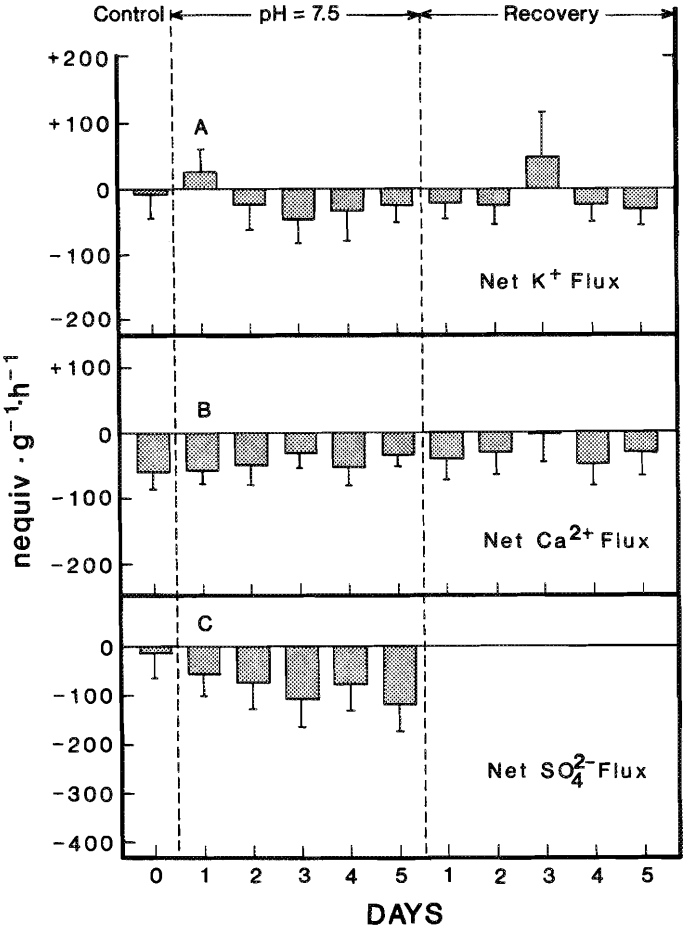

FIG. 7. Net fluxes in *O. propinquus* under maintained control conditions at pH = 7.5 in a regime otherwise duplicating the experimental regime of Fig. 6; n = 26 on days 0–5 for K^+ and Ca^{2+}, n = 5 for SO_4^{2-}, and n = 10 on days 1–5 recovery. Other details as in Fig. 6.

at pH = 7.5, became significantly negative on several days of the acid exposure, averaging ~−60 nequiv·g^{-1}·h^{-1} over the 5-d period (Fig. 6B). During recovery, $J_{net}^{Ca^{2+}}$ returned to the day 0 level. Ca^{2+} balance remained stable in the control animals throughout the experiment.

SO_4^{2-} fluxes were measured in only five or six animals in each group, and no data were obtained from the crayfish followed during the recovery period. At pH = 7.5, $J_{net}^{SO_4^{2-}}$ was close to zero, and there were no significant changes in the control animals (Fig. 6C, 7C). $J_{net}^{SO_4^{2-}}$ became significantly negative (~−250 nequiv·g^{-1}·h^{-1}) throughout the 5 d of acid exposure (Fig. 7C).

Discussion

Control Values

Although haemolymph ionic and acid–base status has not been previously studied in *O. propinquus*, our data for both this species and *O. rusticus* in soft water were generally similar to those reported for *O. rusticus* in hard water (Wilkes and McMahon 1982). We did not see the pronounced metabolic alkalosis and depressed haemolymph Cl^- levels reported for *Procambarus clarki* acclimated to decarbonated water at neutral pH (Morgan and McMahon 1982), perhaps because our water ion levels (especially Na^+ and Ca^{2+}) more closely duplicated those of natural low [HCO_3^-] soft water. Repetitive haemolymph sampling in control animals at neutral pH caused some disturbance of blood acid–base (Fig. 1) and ionic levels (Table 1; Fig. 3) as observed by others (e.g. Truchot 1975; McMahon et al. 1978; Morgan and McMahon 1982; McMahon and Morgan 1983), but these were generally small relative to the experimental effects of acid exposure.

These are the first data on acidic equivalent fluxes in crayfish and on unidirectional exchanges of Na^+ and Cl^- in softwater-acclimated crayfish. Previous studies were relatively short-term single isotope determinations in hard water or abnormal "experimental media," often on ionically depleted animals. Nevertheless, our data on *O. propinquus* are in broad agreement with previous studies on other genera (Shaw 1959, 1960c; Bryan 1960; Kirschner et al. 1973; Ehrenfeld 1974) in showing unidirectional Na^+ fluxes of ~200 nequiv·g^{-1}·h^{-1} and unidirectional Cl^- fluxes approximately threefold greater (Fig. 4, 5). These earlier studies characterized Na^+ and Cl^- uptakes as active, independent, electroneutral exchanges occurring at the gills. Whereas part of Na^+ uptake may be exchanged for acidic equivalents (H^+, NH_4^+) and part of Cl^- uptake for basic equivalents (HCO_3^-, OH^-), a large portion of J_{in} and J_{out} appears to reflect exchange diffusion for both Na^+ and Cl^-.

Our animals were in approximate net Na^+, Cl^-, Ca^{2+}, K^+,

SO_4^{2-}, and acidic equivalent balance at neutral pH (Fig. 4, 5, 6, 7), indicating that resting conditions and complete acclimation to soft water were achieved. Flux rates for most substances were stable under control conditions during the 11-d experiment (Fig. 5, 7), indicating that the experimental protocol itself was not particularly stressful. However, the disturbances in J_{net}^{TA} and J_{net}^{Amm} during the last 3 d (Fig. 5A) may have reflected some physiological deterioration, for by this point the animals had been starved 15–18 d. This may also have caused the gradual fall in unidirectional Cl^- exchanges and the negative J_{net}^{Cl} towards the end of the experiment (Fig. 5C). Alternately it is possible that the slow decline in J_{in}^{Cl} and J_{out}^{Cl} (but *not* the fall in J_{net}^{Cl}) was an experimental artifact. The error in Cl^- flux measurements was greater than in Na^+, for the ^{36}Cl cpm were obtained by subtraction. This, combined with the threefold greater turnover of Cl^-, may have incorporated a small but progressively increasing error in the backflux correction (cf. Maetz 1956) which would have reduced both J_{in}^{Cl} and J_{out}^{Cl} to the same absolute extent. Nevertheless, experimental effects of acid exposure (Fig. 5C) were still discernable.

Influence of Acid Exposure

Our study is the first to combine measures of internal acid–base and ionic status with determinations of the relevant exchanges with the environmental water in crayfish. As such, it allows quantitative analysis of the cause of disturbances seen during acid stress (Table 2), at least within the limitations of intermittent sampling of haemolymph and flux parameters.

The predominant haemolymph response to environmental acidity (pH = 4.0) in softwater-acclimated *O. propinquus* was a large metabolic acidosis (Fig. 1, 2). Similar effects have been seen in *O. rusticus* and *P. clarki* acclimated and tested in hard water at pH = 3.8 (Morgan and McMahon 1982; McMahon and Morgan 1983). In our study, the metabolic acidosis was clearly caused by the massive influx of acidic equivalents (or efflux of basic equivalents, i.e. positive $J_{net}^{H^+}$) from the environment (Fig. 4A), for there was no evidence of lactic acid production. Thus, there was probably no disturbance of O_2 delivery to the tissues. The elevated $J_{net}^{H^+}$ was virtually constant over the 5-d exposure (Fig. 4A), yet the accumulation of acidic equivalents in the haemolymph (i.e. ΔH_b^+) progressively slowed (Fig. 2B). Therefore, most of the acidic equivalent load was quickly moved out of the extracellular fluid volume (ECFV) and buffered in either the intracellular compartment (ICFV) or exoskeleton. Indeed, assuming an ECFV of 0.28 $mL \cdot g^{-1}$ (Kerley and Pritchard 1967), only about 4% of the total load was buffered in the ECFV at the end of 5 d (Table 2). The carapace in crustaceans contains an immense store of basic equivalents, mainly as $CaCO_3$ (Cameron and Wood 1985), so this was likely a major site of buffering. The elevated haemolymph Ca^{2+} levels (Fig. 3; Table 1) and net Ca^{2+} losses to the environment (Fig. 6B; Table 2) support this idea. We suggest that the intracellular compartment of muscle was another important buffer site, for acidic equivalent entry into muscle cells is generally associated with K^+ efflux (Ladé and Brown 1963) as observed here (Fig. 6A; Table 2). It is also possible that some of the observed $J_{net}^{H^+}$ occurred directly at the carapace–water interface, and never entered the haemolymph via the gills. The fact that $J_{net}^{H^+}$ (Fig. 3A) and $J_{net}^{Ca^{2+}}$ (Fig. 6B) returned to normal during recovery before apparent excretion of the total acidic equivalent load (Fig. 4A) suggests that some permanent demineralization of the exoskeleton occurred. This agrees with findings that postmoult calcification is inhibited by low pH in *O. virilis* in soft water (Malley 1980), and that carapace rigidity and Ca^{2+} content are reduced under acidification stress in the wild (France 1983).

TABLE 2. Total net fluxes (nequiv $\cdot g^{-1}$) with the environmental water over 5 d of acid exposure in *O. propinquus*, partitioned between the extracellular fluid volume (ECFV) and intracellular fluid volume (ICFV)/exoskeleton compartments.

	Total	ECFV[a]	ICFV/exoskeleton
H^+	+54 800	+2 300	+52 500
Na^+	−13 200	−11 300	−1 900
Cl	−5 300	−7 300	+2 000
K^+	−10 900	0	−10 900
Ca^{2+}	−9 100	+1 400	−10 500
Net charge[b]	+26 900	−300	+27 200

[a]Assuming ECFV = 28% body weight (Kerley and Pritchard 1967).
[b]Net charge = $(H^+ + Na^+ + K^+ + Ca^{2+}) - Cl^-$.

The elevation of Pa_{CO_2} during the first 2 d (Fig. 1C) also contributed to haemolymph acidosis and could have resulted from hypoventilation or a thickened diffusion barrier at the gills. Applying the analysis of Wood et al. (1977), increased Pa_{CO_2} accounted for 30–40% of the total pHa depression on days 1 and 2 and was negligible thereafter (Fig. 2A). The very low Pa_{CO_2} on day 12 served to reverse the metabolic pHa depression by ~25%, possibly a last ditch attempt to stave off fatal acidosis by hyperventilation. Interestingly, while *O. rusticus* showed a similar Pa_{CO_2} elevation, *P. clarki* exhibited decreased Pa_{CO_2} throughout 4 d of acid exposure (Morgan and McMahon 1982; McMahon and Morgan 1983).

While haemolymph electrolytes were also disturbed by low pH (Fig. 3; Table 1), the Na^+ and Cl^- depressions (~20%) were not as serious as the acid–base effects (70% loss of HCO_3^-, tripling of free H^+ concentration; Fig. 1). At least qualitatively, this was similar to the situation in *O. rusticus* and *P. clarki* in hard water (Morgan and McMahon 1982; McMahon and Morgan 1983). Losses of Na^+ and Cl^- from the haemolymph were almost entirely explained by losses to the environment, and net Na^+ and Cl^- fluxes with the ICFV or carapace were small (Table 2). In contrast, net Ca^{2+} and K^+ losses to the water (Fig. 6A, 6B) occurred entirely from outside the ECFV (Table 2), likely from the carapace and muscle compartments, respectively.

The unidirectional Na^+ and Cl^- flux measurements showed that net losses were initially due to modest inhibitions of $J_{in}^{Na^+}$ (by ~50%; Fig. 4B) and $J_{in}^{Cl^-}$ (by ~30%; Fig. 4C), while efflux rates were unaffected. We are aware of no previous work on low pH effects on Cl^- exchanges in freshwater crustaceans. However, the one previous study on Na^+ balance at low pH in crayfish (Shaw 1960b) showed similar effects (inhibited $J_{in}^{Na^+}$, unchanged $J_{out}^{Na^+}$). At pH = 4.0 in our experiments, H^+ ions were available in the external medium at half the concentration of Na^+ ions, so that 50% inhibition of $J_{in}^{Na^+}$, as well as some of the increase in $J_{net}^{H^+}$, could have resulted from H^+ versus Na^+ competition for a common carrier. The 30% inhibition of $J_{in}^{Cl^-}$ is more difficult to explain, but has been commonly observed in fish and amphibians (Wood and McDonald 1982; McDonald 1983a); it could reflect conformational changes in the carrier and/or the reduction of internal HCO_3^- levels (Fig. 1B) impeding Cl^-/HCO_3^- exchange. During continued acid exposure,

Cl^- fluxes remained stable at the day 1 level (Fig. 4C) but both $J_{in}^{Na^+}$ and $J_{out}^{Na^+}$ progressively increased (Fig. 4B). This could be explained by an increasing exchange diffusion component rather than recovery of Na^+ versus acidic equivalent exchange because $J_{out}^{Na^+}$ remained elevated upon return to neutral pH, whereas $J_{in}^{Na^+}$ showed a further immediate increase. The latter would represent restoration of Na^+ versus acidic equivalent exchange which in turn would restore positive $J_{net}^{Na^+}$, while the exchange diffusion component gradually declined over the 5-d recovery period.

Comparison with Fish

While the responses of *O. propinquus* to low pH were superficially similar to those of fish, their detailed nature was very different. In the gills of the rainbow trout (*Salmo gairdneri*), $J_{net}^{H^+}$ is constrained by the difference between strong cation (mainly Na^+) and strong anion (mainly Cl^-) fluxes (McDonald 1983b; Wood et al. 1984) as predicted by the strong ion difference concept (Stewart 1978) and the demands of electroneutrality. During acid stress in hard water (high $[Ca^{2+}]$) the gill epithelium becomes more permeable to Na^+ than to Cl^-, so positive $J_{net}^{H^+}$, acidosis, and Na^+ loss in excess of Cl^- loss occur (McDonald et al. 1980; McDonald and Wood 1981; Wood and McDonald 1982; McDonald 1983a, 1983b; McDonald et al. 1983). In contrast, in soft water (low $[Ca^{2+}]$), acid stress elevates Na^+ and Cl^- permeabilities by greater but approximately equal amounts, so $J_{net}^{H^+}$ is negligible, acidosis does not occur, but large equimolar losses of Na^+ and Cl^- result in rapid mortality. Thus, the response of the crayfish in *soft* water (highly positive $J_{net}^{H^+}$, severe acidosis, relatively small Na^+ loss considerably in excess of Cl^- loss) resembled that of the trout in *hard* water. Perhaps the higher Ca^{2+} levels in the blood and tissues of crayfish are responsible for a "hard-water type" response in soft water.

While the highly positive $J_{net}^{H^+}$ was correlated with a loss of strong cations (Na^+, K^+, and Ca^{2+}), far in excess of strong anion (Cl^-) as predicted by theory, a considerable charge imbalance remained, a deficit equal to ~50% of $J_{net}^{H^+}$ over 5 d (Table 2). This has not been seen in the trout studies. This deficit was associated entirely with the ICFV or exoskeleton, for good charge balance was achieved in the ECFV (Table 2). Although SO_4^{2-} is reportedly impermeant at the gills of crayfish (Shaw 1960a; Ehrenfeld 1974), we suspected that a direct entry of SO_4^{2-} into the carapace (in association with H^+ and effectively in exchange for CO_3^{2-}) might be responsible. While SO_4^{2-} fluxes were measured in only a few animals (Fig. 6C, 7C) and therefore the data not included in Table 2, this was clearly not the explanation. SO_4^{2-} was lost in significant amounts during acid exposure, which would exacerbate rather than reduce the charge imbalance of Table 2. The explanation remains unknown.

Further differences from fish occurred in the flux effects. In both rainbow trout (McDonald et al. 1983) and white sucker (*Catostomus commersoni*; Hōbe et al. 1984), pH ≅ 4.0 caused a greater immediate inhibition (60–95%) of both $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$, and very large diffusive increases in $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$. The latter were the major causes of ion loss during acute exposure. During continued acid stress, J_{out} values returned to control levels without recovery of J_{in} values, so the inhibition of active uptake was the major source of ion loss over the longer term in fish. This is very different from the crayfish where inhibitory effects on J_{in} components predominated throughout (Fig. 4B, 4C). While interpretation is clouded by the possibly changing role of exchange diffusion, this at least indicates that permeability of the gills to passive effluxes in crayfish is much more acid resistant than in fish. Furthermore, in fish, positive $J_{net}^{H^+}$ was attenuated during continued acid exposure (McDonald 1983b; McDonald et al. 1983; Hōbe et al. 1984). That this did not occur in crayfish (Fig. 4A) again suggests direct H^+ penetration of the carapace. Finally, unlike fish, acid exposure had no effect on J_{net}^{Amm} in *O. propinquus*, which suggests that Na^+ versus NH_4^+ exchange is of lesser importance in crayfish than in fish (cf. Wright and Wood 1985), in agreement with previous studies (Kirschner et al. 1973; Ehrenfeld 1974). While much further work is needed to understand the mechanisms behind acid stress responses in crayfish, it is already clear that extrapolation from fish data will be of only very limited help.

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