THE INFLUENCE OF CALCIUM ON THE PHYSIOLOGICAL RESPONSES OF THE RAINBOW TROUT, *SALMO GAIRDNERI*, TO LOW ENVIRONMENTAL pH

By D. G. McDONALD, H. HÖBE* AND C. M. WOOD

Department of Biology, McMaster University,
Hamilton, Ontario, Canada L8S 4K1

SUMMARY

The physiological responses of 1- to 2-year-old rainbow trout to low pH are dependent on the environmental calcium concentration. Trout, maintained for 5 days in moderately hard water ($[Ca^{2+}] = 1\cdot6 - 2\cdot7$ m-equiv/l) at a mean pH of $4\cdot3$, developed a major blood acidosis but exhibited only a minor depression in plasma ion levels. In acidified soft water ($[Ca^{2+}] = 0\cdot3$ m-equiv/l), only a minor acidosis occurred, but plasma ion levels fell and there were substantially greater mortalities. Lethal bioassays performed on fingerling trout over a range of pH levels ($3\cdot0-4\cdot8$) revealed an important influence of external $[Ca^{2+}]$ on resistance to acid exposure. Terminal physiological measurements on adult fish succumbing to low pH in soft water indicate the singular importance of iono-regulatory failure as the toxic mechanism of action under these circumstances.

INTRODUCTION

Acid precipitation resulting from industrial emissions of sulphur and nitrogen oxides now occurs over wide areas of Northern Europe and North America (Cogbill & Likens, 1974; Odén, 1976; Dillon *et al.* 1978). Lakes susceptible to acid input are typically soft waters with low ionic strength and low buffer capacity. Extensive surveys (Harvey, 1975; Schofield, 1976; Leivestad *et al.* 1976) have documented the progressive acidification of many such lakes and the concomitant loss of fish populations. Fish stocks are affected if the pH is less than $5\cdot5$ (Leivestad *et al.* 1976) and lakes below pH $4\cdot3$ are normally completely devoid of even the most acid-tolerant species (Harvey, 1979). While decreased recruitment of young fish has been widely cited as the primary factor in this disappearance (e.g. Schofield, 1976), massive fish kills brought on by episodic excursions of water pH to toxic levels (pH $\leqslant 4\cdot3$) have been reported in a number of instances (Jenson & Snekvik, 1972; Leivestad & Muniz, 1976; Harvey, 1979).

Several studies have been made of the physiological mechanisms of acid toxicity. Major disturbances in blood oxygen transport (Vaala & Mitchell, 1970; Packer,

* Present address: Department of Biology, University of Calgary, Calgary, Alberta, Canada, T2N 1N4.

1979), blood acid–base state (Lloyd & Jordan, 1964; Packer & Dunson, 1970, 1972; Neville, 1979; Packer, 1979) and ionic balance (Packer & Dunson, 1970, 1972; Mudge & Neff, 1971; Leivestad & Muniz, 1976; McWilliams & Potts, 1978) have now been reported, but the primary cause of death remains unknown. A major complicating factor is that the ionic concentrations of test waters have varied from those typical of acidified soft water lakes (e.g. Leivestad *et al.* 1976) to levels at least 10-fold higher (e.g. Neville, 1979). In both field and laboratory studies (Packer & Dunson, 1972; Leivestad *et al.* 1976) increased ion levels have been shown to improve the survival of fish exposed to low pH. Ameliorative effects apparently result from the elevation of either NaCl (Packer & Dunson, 1972; Leivestad *et al.* 1976) or Ca and Mg salts (Leivestad *et al.* 1976). Possibly the nature of the mechanism of acid toxicity also varies with the nature of the ionic environment.

In the present investigation, we have examined the physiological responses of the rainbow trout during chronic exposure to low environmental pH in hard and soft water. Repetitive blood sampling from chronically catheterized animals has been employed to monitor blood acid–base status (arterial pH, $[HCO_3^-]$ and P_{CO_2}), ionic status (plasma $[Na^+]$, $[Cl^-]$, $[K^+]$, $[Ca^{2+}]$) and the extent of anaerobic metabolism (blood lactate levels), the latter serving as an index of the adequacy of blood O_2 transport.

MATERIALS AND METHODS
Experimental animals

Rainbow trout (*Salmo gairdneri*) of both sexes were obtained from Spring Valley Trout Farm, Petersburg, Ontario and held at least 1 week prior to experimentation in large fibreglass tanks continuously supplied with well-aerated, dechlorinated tap water at 7–12 °C. One- to two-year-old trout (90–442 g) were used in physiological studies, and fingerlings (2–5 g) in toxicity tests. The animals were acclimated for 2 weeks to water of the ionic composition (see below) and temperature (11 ± 2 °C) appropriate for the experimental tests. The animals were fed regularly with commercial trout pellets but starved during the last week of acclimation and the subsequent experimental period.

To allow chronic blood sampling in the physiological studies, trout were anaesthetized in MS-222 (1:10000 dilution in the acclimation water) and surgically implanted with dorsal aortic catheters (Smith & Bell, 1964). The fish were allowed to recover in acclimation water for 24–48 h. Control (i.e. day 0) blood samples were then drawn from each fish and analysed for acid–base state and ion levels as described below. Subsequently fish were either exposed to acidified water (pH 4·0–4·5; experimental series) or allowed to remain in the acclimation water (pH 7·0–7·5; control series). Blood samples were drawn daily under each condition for at least 5 subsequent days. The control series were employed to separate the effects of the experimental protocol alone (e.g. catheterization, repetitive blood sampling, acclimation time) from the effects of acid exposure on acid–base state and ion levels.

Test conditions

All physiological experiments were conducted in temperature-controlled (11 ± 1 °C) recirculating water systems, each supplying eight darkened plexiglass chambers (2 l volume) in which individual fish were isolated. Each chamber received a flow of at least 500 ml/min and separate aeration. The recirculating systems consisted of either one 300 l reservoir (control experiments) or two 100 l reservoirs (acid exposure experiments). In the latter, one reservoir supplied water at normal pH (pH 7·0–7·5), while the other supplied acidified water (initially at pH 4·0, see below). The switch-over from one reservoir to the other could be effected without disturbance to the fish. The systems contained no internal metal components, so as to minimize the risk of heavy metal poisoning from acid leaching of metal ions. Heavy metal ion levels were determined on acidified water resident in the recirculating system for 6 days (Table 1). With the exception of cadmium, the concentrations were below the water quality objectives (International Joint Commission, 1976). The cadmium level, while above the objective, was less than 6% of the 10-day lethal threshold concentration for trout at 12 °C (Roch & Maly, 1979).

In all series, both control and experimental, water was decarbonated prior to use in order to avoid possible complicating effects of high P_{CO_2} (Lloyd & Jordan, 1964; Neville, 1979) that might otherwise accompany acid titration of water bicarbonate. Decarbonation was effected by HCl acidification to pH 2·5, 24 h aeration, and back titration to the appropriate pH with NaOH (KOH in the high [Ca²⁺], low [salt] water series). Water P_{CO_2} was always less than 1 mmHg during the experiments. HCl was used for water acidification in all experiments rather than H_2SO_4, the more common mineral acid pollutant in the wild, to avoid possible toxic effects of sulphate anion (Maetz, 1973). A nominal pH of 4·0 was chosen for the experimental series. The median survival time of rainbow trout in soft water is in excess of 5 days at this pH (Leivestad *et al.* 1976), thus allowing adequate time in which to examine the development of deleterious physiological effects. The pH of the acidified water rose when being circulated through the fish chambers. To counter this, the water pH was returned to 4·0 at twice-daily intervals during the experiment by addition of HCl. The actual mean pH over the duration of the experiments was about 4·3 (Table 1).

Experiments were carried out in either 'hard' water, 'soft' water or high [Ca²⁺], low [salt] water. The chemical compositions of these waters are listed in Table 1. 'Hard' water was dechlorinated Hamilton, Ontario tap water. 'Soft' water was prepared by a 10-fold dilution of tap water with distilled water, and contained ions at concentrations approximating those found in acidified natural waters (Table 1). The mean effective dilution was slightly less than 10-fold for [Na⁺] and [Ca²⁺], and non-existent for [K⁺] because of the continual addition of these ions to the water by the fish during the course of an experiment (Table 1). High [Ca²⁺], low [salt] water was prepared by the addition of Ca(NO₃)₂ to soft water so as to raise [Ca²⁺] to levels approximating those in hard water (Table 1). Four experimental treatments were employed:

Acclimated hard water. Fish were acclimated and tested in hard water. Control ($N = 8$) and experimental ($N = 21$) series were run.

Table 1. *Chemical composition of water employed in acid-exposure and control experiments compared with George Lake, Ontario, a typical acidified soft water lake (Beamish et al., 1975). Values are means with range in brackets. Ions are in m-equiv/l*

	'Hard' water		'Soft' water*		High [Ca^{2+}], low [salt] water (acid)	George Lake
	Control	Acid	Control	Acid		
pH	7·3 (7·2–7·7)	4·2 (4·0–4·5)	7·3 (7·2–7·5)	4·3 (4·0–4·5)	4·4 (4·0–5·3)	4·8–5·3
Cl^-	4·5 (3·7–4·9)	5·6 (4·8–6·9)	0·4 (0·3–0·4)	1·0 (0·3–1·9)	1·1 (0·5–1·7)	0·03 (0·02–0·05)
Na^+	2·9 (2·4–3·3)	3·5 (3·2–3·7)	0·4 (0·3–0·6)	0·4 (0·1–0·7)	0·5 (0·4–0·6)	0·03 (0·03–0·04)
K^+	0·05 (0·04–0·06)	0·09 (0·04–0·15)	0·06 (0·04–0·08)	0·05 (0·01–0·1)	0·2 (0·1–0·2)	0·01 (0·01–0·02)
Ca^{2+}	2·3 (1·7–2·9)	1·6 (1·5–1·7)	0·4 (0·1–0·5)	0·3 (0·2–0·4)	2·7 (2·2–3·3)	0·2 (0·1–0·2)

• Soft water values are pooled means (ranges) for the acclimated and unacclimated series. Trace metals (in parts per billion) in recirculating acid water system after 6 days (water quality objective (IJC, 1976) in parentheses): cadmium, 1·6 (0·2); chromium, 10 (50); copper, 4 (5); iron, 12 (300); lead, 4 (25); nickel, 21 (25); zinc, 24 (30).

(ii) *Acclimated soft water*. Fish were acclimated and tested in soft water. Control (N = 6) and experimental (N = 8) series were run.

(iii) *Unacclimated soft water*. Fish were acclimated to hard water but were tested in soft water. Control (N = 7) and experimental (N = 8) series were run.

(iv) *Acclimated high* [Ca^{2+}], *low* [*salt*] *water*. An experimental series only (N = 7) was run.

Analytical procedures

Blood samples ($\sim$ 0·6 ml) were drawn anaerobically using chilled, gas-tight Hamilton syringes. An equal volume of heparinized (100 i.u./ml) Cortland saline (Wolf, 1963) was then returned to the animal via the catheter. Each blood sample was analyzed for the following (except where noted in Results): haematocrit, pHa, total CO_2 content (C_{a, CO_2}, whole blood and plasma), lactate, plasma [Na^+], plasma [Cl^-], plasma [K^+], plasma [Ca^{2+}] and plasma [protein].

Blood pH was determined on 20 μl aliquots injected into a Radiometer microelectrode connected to a Radiometer PHM-72 acid–base analyzer and thermostatted to the experimental temperature. Before each measurement the pH electrode calibration was checked with Radiometer precision buffers (S 1500 and S 1510). Haematocrits were measured by centrifuging 80 μl blood samples in heparinized capillary tubes at 5000 g for 5 min. Blood and plasma C_{CO_2}s (50 μl samples) were assayed by the micro-method of Cameron (1971), using a teflon membrane on the P_{CO_2} electrode for greater stability and bracketing each unknown with $NaHCO_3$ standards to increase the precision of this technique. Lactate analyses were performed on 150 μl of blood immediately deproteinized in 300 μl of ice-cold perchloric acid (8%, w/v) and then centrifuged at 5000 g for 10 min. The supernatant was analyzed 1-lactate with Sigma reagents (Sigma, 1976). Chloride determinations were made on 20 μl plasma samples using a Radiometer CMT-10 chloride titrator. Sodium (20 μl plasma sample), potassium (100 μl) and calcium (100 μl) concentrations were determined after dilution (1:500, 1:30, and 1:100, respectively) on an Eel (Na^+ and K^+) or Coleman 20 (Ca^{2+}) flame photo-meter. Appropriate swamping was employed to eliminate the interfering effect of Na^+ on Ca^{2+} and K^+ emissions. Plasma samples (10 μl) were analysed for plasma protein concentration using a Goldberg refractometer (American Optical).

Calculations

1. [HCO_3^-] and $P_{a, CO2}$

Plasma and whole blood [HCO_3^-] and P_{a, CO_2} were calculated from measurements of C_{CO_2} and pH in whole blood and plasma, using the Henderson–Hasselbalch equation:

$$pH = pk_1' + \log \left(\frac{HCO_3^-}{\alpha CO_2 \cdot P_{CO_2}} \right), \tag{1}$$

where pk_1' is the apparent first dissociation constant of H_2CO_3 and αCO_2 is the CO_2 solubility coefficient in plasma. Values for αCO_2 corrected to the experimental temperature, and values for pk_1' corrected to both experimental temperature and plasma pH were obtained from a table of Severinghaus (1965).

2. ΔH^+_b

The daily change in the quantity of H^+ ions (in m-equiv/l blood) added to blood buffers by non-respiratory (i.e. non-volatile) acids (ΔH^+_b) was calculated from procedures of Wood, McMahon & McDonald (1977) and McDonald, Boutilier & Toews (1980), according to the formula:

$$\Delta H^+_b = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pH_1 - pH_2), \tag{2}$$

where β is the slope of the blood non-bicarbonate (i.e. protein) buffer line ($\Delta[HCO_3^-]/\Delta pH$) and the subscripts 1 and 2 refer to whole blood $[HCO_3^-]$ and plasma pH measurements made at daily intervals.

In preliminary experiments β was determined (using procedures outlined by Wood et al. 1977) by in vitro CO_2 titration of blood samples drawn via chronic dorsal aortic catheters from trout ($N = 13$) exhibiting a range of in vivo haematocrits from 6·5 to 33·0%. From these data a significant linear relationship ($P < 0.01$) between haematocrit (h) and β was found as described by the regression equation:

$$\beta = -24.60h - 3.97 \tag{3}$$

($r = 0.773$, S.E. slope = 5·60, S.E. intercept = 0·53). Thus, ΔH^+_b can be calculated for an individual fish over, for example, the first day of acid exposure by substitution of the following values in equation (2): 1 = pH and $[HCO_3^-]$ measurements for that fish on day 0, 2 = measurements on day 1, and β = value calculated from equation (3) using the haematocrit value (expressed as a decimal) on day 0.

3. Blood volumes

Final blood volumes in acid-exposed fish were estimated from the changes in haematocrit and plasma protein concentration which occurred over the course of the experiment (cf. Fig. 10). The calculations (see below) assume that the initial blood volume was 5·0 ml/100 g (Stevens, 1968) in all fish, that the blood volume was unaffected by repetitive blood sampling and that the amounts of erythrocytes and plasma protein lost from the blood per 100 g due to sampling were the same for acid-exposed and control fish. The last assumption is true only when the body weights of control and acid-exposed fish are similar. This condition existed for all groups except the acclimated hard-water group exposed to acid. The overall mean weight for the former groups was 305 ± 8 g and there were no significant differences among the groups, whereas the body weights for the latter were significantly lower (140 ± 11 g). Therefore the data from these fish were excluded from the analysis.

The final blood volume (BV_f) in acid-exposed fish was calculated from initial and final haematocrits (h_i and h_f) according to the formula:

$$BV_f = \frac{BV_i \cdot h_i - R_{ev}}{h_f}, \tag{4}$$

where R_{ev} is the mean reduction in volume of erythrocytes per 100 g in control fish calculated according to the formula:

$$R_{ev} = \frac{\Sigma BV_i(h_i - h_f)}{n},$$

where $BV_i = 5.0$ ml/100 g.

Similarly, BV_f in acid-exposed fish was calculated from initial and final plasma protein concentrations (P_i and P_f) according to the formula:

$$BV_f = \frac{P_i BV_i(1-h) - R_{prot}}{P_f(1-h_f)},$$ (6)

where R_{prot} is the mean reduction in plasma protein content in control fish (g protein/ 100 g fish) calculated according to the formula:

$$R_{prot} = \frac{\Sigma BV_i(P_i(1-h_i) - P_f(-h_f))}{n}.$$ (7)

Statistical analyses

Means ± one S.E.M. are reported throughout. These means (Figs. 1–10) exclude values from fish that succumbed during the 5-day acid exposure. The final values recorded prior to death for these fish are presented separately (Table 3). Significant differences ($P < 0.05$) within each acid series were tested with Student's two-tailed t test (paired design) using each fish for its own 'control' values (the day 0 values). Significant differences ($P < 0.05$) among acid series were tested with Student's two-tailed t test (unpaired design).

Toxicity tests

The relative toxicity of HCl in hard ($[Ca^{2+}] = 3.3$ m-equiv/l) and soft ($[Ca^{2+}] = 0.2$ m-equiv/l) water was assessed in the classical manner (Sprague, 1969) by 7-day lethality tests on fingerling trout (2–5 g) acclimated to water of the appropriate composition. Water was prepared as in the physiological studies. The tests were conducted in 80 l polyethylene recirculatory systems. A range of pH levels from 3.0 to 4.8 in 0.2 unit increments was employed; pH control was accurate within ±0.05 unit up to 4.4, and ±0.1 unit at 4.6 and 4.8. The fish were tested at each pH, and individual mortality times recorded for each fish. Median lethal times, 95% confidence limits, and significance of differences ($P < 0.05$) were estimated by log-probit analysis (Litchfield, 1949).

RESULTS

Acclimated hard water

In hard-water-acclimated trout, the five-day exposure to low external pH resulted in a fall in arterial pH (Fig. 1 C) and $[HCO_3{}^-]$ (Fig. 1 B). The acidosis was apparently not due to the endogenous generation of respiratory or metabolic acids since there was no concomitant change in P_{a, CO_2} and lactate levels (Fig. 1 A, D), but rather represented an invasion of the extracellular fluid compartment by external H^+ ions.

In Fig. 1 C it can be seen that the acidosis was virtually fully developed by day 2. This may also be seen in the calculated $\Delta H^+{}_b$ (Fig. 2 A) and was further borne out by measurements made on four fish until day 9. Net $\Delta H^+{}_b$ was 5.6 ± 0.7 m-equiv/l by day 2, and 5.9 ± 0.7 m-equiv/l by day 5 (Fig. 2 A). By day 9 net $\Delta H^+{}_b$ had further ▇reased by $23 \pm 6\%$ only.

Accompanying this blood acid–base disturbance was a relatively minor plasma
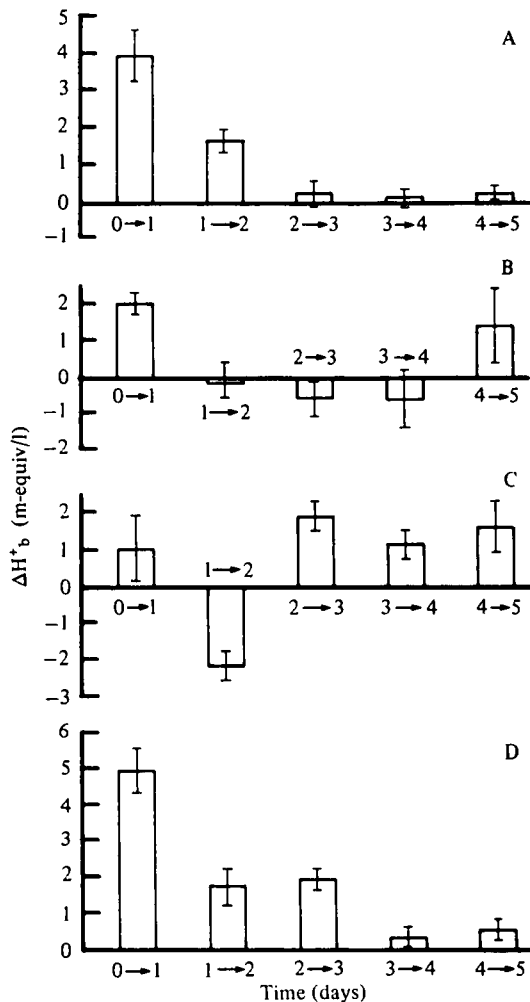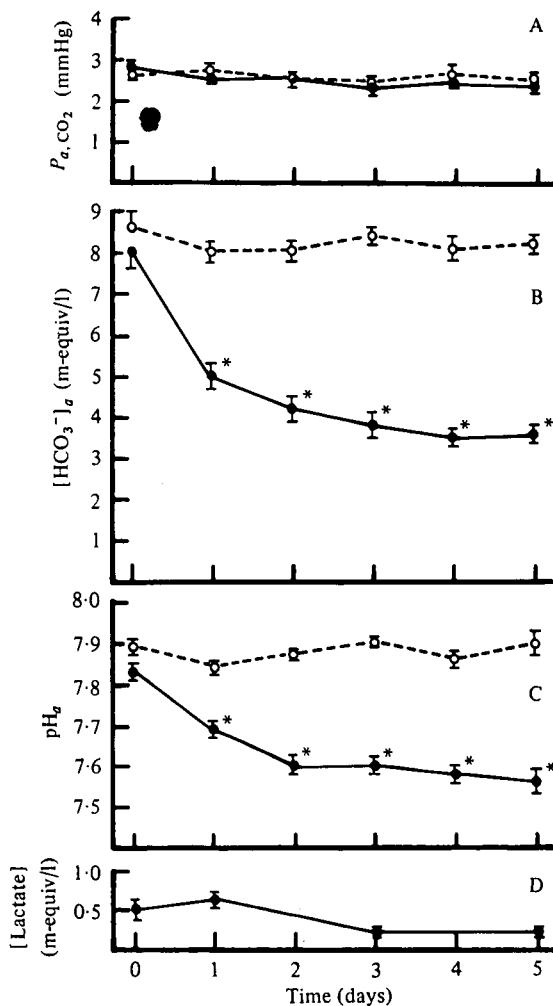
Fig. 1. Blood acid–base state (means ± one S.E.M.) in rainbow trout acclimated to hard water. (A) Arterial CO$_2$ tension. (B) Arterial bicarbonate concentration. (C) Arterial pH. (D) Arterial lactate. Animals either held at neutral pH throughout (controls: O-----O, N = 8) or exposed to low pH (●—●, N = 17) following the day 0 blood sample. Asterisks indicate significant difference (P < 0·05) from day 0 values (by paired t test).

Fig. 2. Net daily acid accumulation in blood (ΔH^+_b, means ± one S.E.M.) of rainbow trout in the following. (A) Acidified hard water (N = 17). (B) Acidified soft water, trout acclimated to soft water for 2 weeks prior to low pH exposure (N = 7). (C) Acidified soft water, trout not acclimated (N = 5). (D) Acidified high [Ca^{2+}], low [salt] water (N = 7).

ionic disturbance (Fig. 3). There was a significant fall in plasma [Na$^+$] which was apparently complete by day 3. Plasma [K$^+$] showed only a very slight increase, significant on day 3 only, while plasma [Ca^{2+}] and [Cl$^-$] showed no significant variation.

The hard water control series clearly indicated that the experimental procedure had no effect on acid–base (Fig. 1) or ionic parameters (Fig. 3).
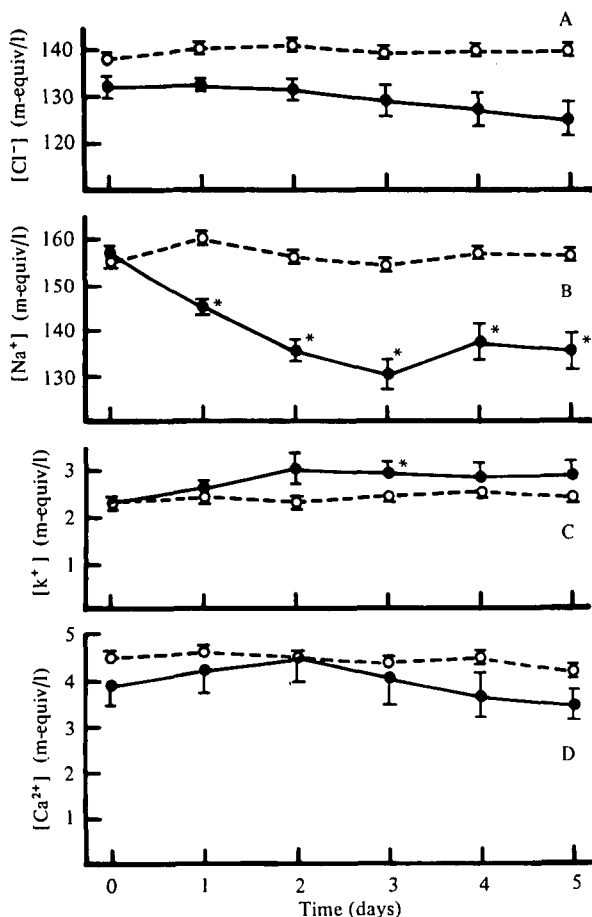
Fig. 3. Plasma ion levels (means ± one S.E.M.) in rainbow trout acclimated to hard water. Animals either held at neutral pH throughout (controls, O-----O, $N = 8$) or exposed to low pH (●—●, $N = 7$) following the day o blood sample. Asterisks indicate significant difference ($P < 0.05$) from day o values (by paired t test).

Acclimated soft water

In soft-water-acclimated fish, day o values for P_{a, CO_2}, $[HCO_3^-]$ and $[K^+]$ were significantly lower than in fish acclimated to hard water (Table 2). However, pH_a was unaffected, as were plasma Na^+, Cl^- and Ca^{2+} levels (Table 2) despite the much lower concentrations of these ions in the water (Table 1).

Exposure to low external pH resulted in only a small drop in pH_a (Fig. 4C); a third that in the hard-water-acclimated fish. Furthermore, some recovery from this mild acidosis was indicated, for day 4 and day 5 values were not significantly different from the pH_a at day o. Plasma $[HCO_3^-]$ showed a small significant decline on day 1 but was otherwise unchanged until day 5 when there was also a rise in blood lactate (Fig. 4D). P_{a, CO_2} was unaffected (Fig. 4A).

The net ΔH^+_b by day 5 (i.e. the sum of the daily values in Fig. 2B) was 2.0 ± 0.9 m-quiv/l. A large fraction of this quantity can be accounted for by the increase in

Table 2. *Initial blood parameters (i.e. day 0 at neutral pH) in rainbow trout under the four treatment conditions. Means ± one* S.E.M. *(N)*

	Acclimated hard water*	Acclimated soft water*	Unacclimated soft water*	High [Ca²⁺], low [salt] water
pH$_a$	7·851 ± 0·015 (25)	7·874 ± 0·029 (13)	7·905 ± 0·030 (12)	7·849 ± 0·026 (7)
P$_{a,CO_2}$ (m-equiv/l)	2·71 ± 0·15 (25)	2·30 ± 0·07† (13)	2·07 ± 0·16† (12)	2·84 ± 0·11‡ (7)
[HCO$_3$⁻] (m-equiv/l)	8·16 ± 0·26 (25)	7·15 ± 0·37† (13)	7·28 ± 0·38 (12)	8·42 ± 0·40‡ (7)
[Na⁺] (m-equiv/l)	156·1 ± 0·6 (15)	154·1 ± 1·0 (13)	142·4 ± 1·7†‡ (12)	150·8 ± 1·3†‡ (7)
[Cl⁻] (m-equiv/l)	135·4 ± 0·6 (15)	137·4 ± 1·1 (13)	134·3 ± 1·1‡ (12)	131·5 ± 0·9†‡ (7)
[K⁺] (m-equiv/l)	2·36 ± 0·05 (15)	2·09 ± 0·06† (13)	2·33 ± 0·06‡ (12)	2·21 ± 0·08 (7)
[Ca²⁺] (m-equiv/l)	4·10 ± 0·11 (15)	4·23 ± 0·12 (13)	4·04 ± 0·13 (12)	3·95 ± 0·18 (7)
Plasma protein (g/100 ml)	2·7 ± 0·1 (8)	2·8 ± 0·1 (7)	—	3·1 ± 0·1† (7)
Lactate (m-equiv/l)	0·5 ± 0·1 (7)	0·5 ± 0·1 (7)	—	—

* Day 0 values for control and experimental groups combined.
† Significantly different (*P* < 0·05) from corresponding acclimated hard water value.
‡ Significantly different (*P* < 0·05) from corresponding acclimated soft water value.

blood lactate during this period (1·30 ± 0·35 m-equiv/l; Fig. 4D). Thus the net ΔH^+_b of external origin by day 5 (i.e., Δ lactate subtracted) amounted to 0·69 ± 0·71 m-equiv/l, a value only 12% of that in acclimated hard water fish.

Plasma [Na⁺] and [Cl⁻] both declined progressively during the acid exposure in soft water (Fig. 5) to lower levels than the corresponding values in hard water (Fig. 3). The increase in [K⁺] was also more pronounced (Fig. 5C) than in hard water (Fig. 3C). Plasma [Ca²⁺] declined significantly from day 2 onwards, but a similar trend was seen in the control group (Fig. 5D). There were no other significant changes within the control group (Figs. 4, 5).

Unacclimated soft water

These experiments were designed to test whether the influence of soft water on the effects of acid exposure, as observed above, would be accentuated or reduced by acute exposure to soft water. A second objective was to determine the time course of soft-water acclimation, by looking for changes in the control series relative to the two preceding control series (in which all parameters, with the possible exception of Ca²⁺, were unaffected by repetitive sampling).

The day 0 values for acid–base parameters in soft-water-unacclimated fish closely resembled those in soft-water-acclimated animals (Table 2). However, plasma [Na⁺] and [Cl⁻] were both significantly depressed and [K⁺] significantly elevated. Plasma [Ca²⁺] was unaffected (Table 2).

Environmental acid caused no significant blood acidosis over 5 days in these unacclimated fish (Fig. 6C). Indeed, on day 2 they exhibited a significant metabolic alkalosis relative to controls (Fig. 6C). However, [HCO₃⁻] did fall slightly on day
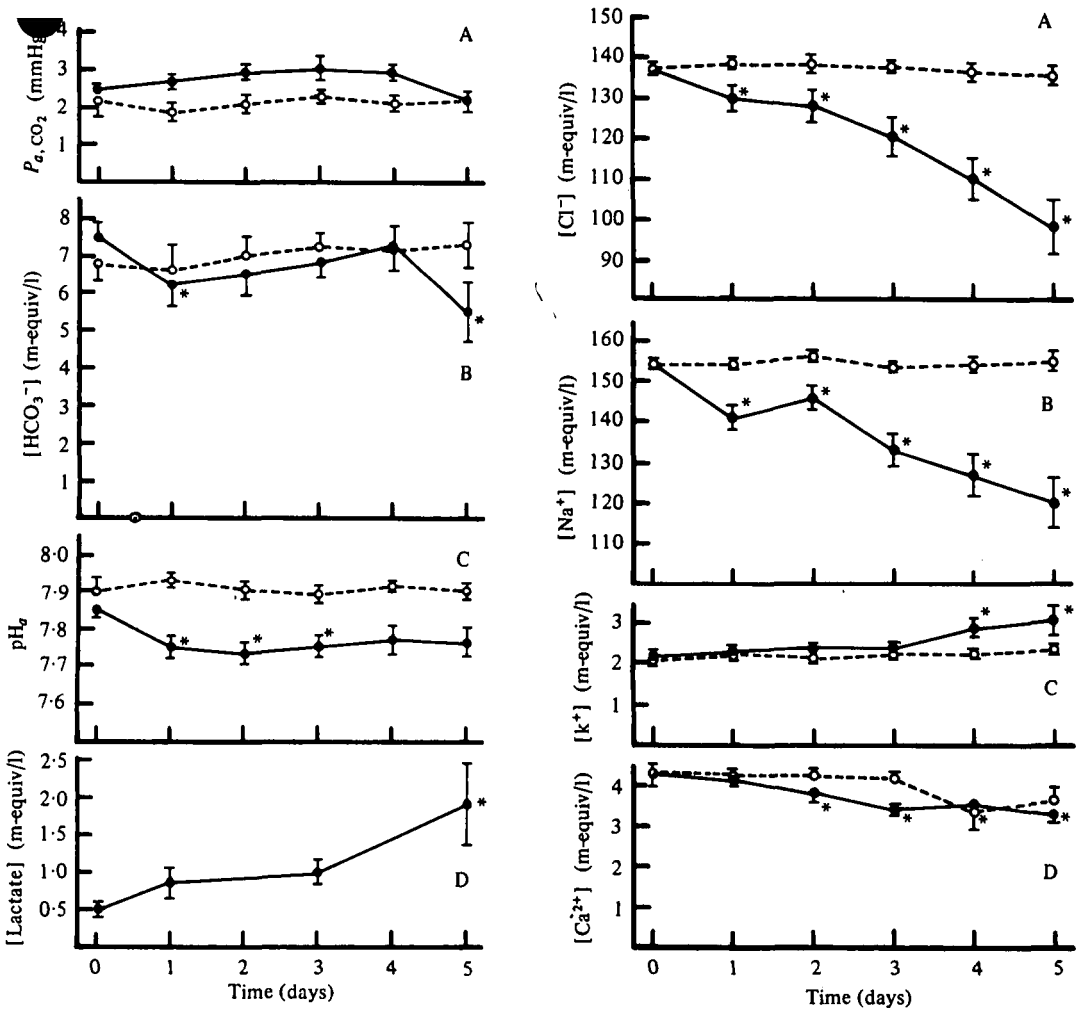
Fig. 4. Blood acid–base state (means ± one S.E.M.) in rainbow trout acclimated to soft water. (A) Arterial CO_2 tension. (B) Arterial bicarbonate concentration. (C) Arterial pH. (D) Arterial lactate. Animals either held at neutral pH throughout (controls, O-----O, N = 6) or exposed to low pH following the day o blood sample (●—●, N = 7). Asterisks indicate significant difference (P < 0·05) from day o values (by paired t test).

Fig. 5. Plasma ion levels (means ± one S.E.M.) in rainbow trout acclimated to soft water. Animals either held at neutral pH throughout (controls, O-----O, N = 6) or exposed to low pH (●—●, N = 7) following the day o blood sample. Asterisks indicate significant difference (P < 0·05) from day o values (by paired t test).

and markedly on day 5 (Fig. 6 B); pH_a was maintained by a corresponding drop in P_{a, CO_2}. Overall, the pattern of $\Delta H^+{}_b$ variation was similar to that in acclimated soft-water fish (Fig. 2 B, C).

Plasma [Na$^+$] and [Cl$^-$] fell markedly during acid exposure (Fig. 7 A, B) and the day 5 values were virtually identical to those in soft-water-acclimated fish (Fig. 7 A, However, the decrease in [Na$^+$] was perhaps underestimated, since the level
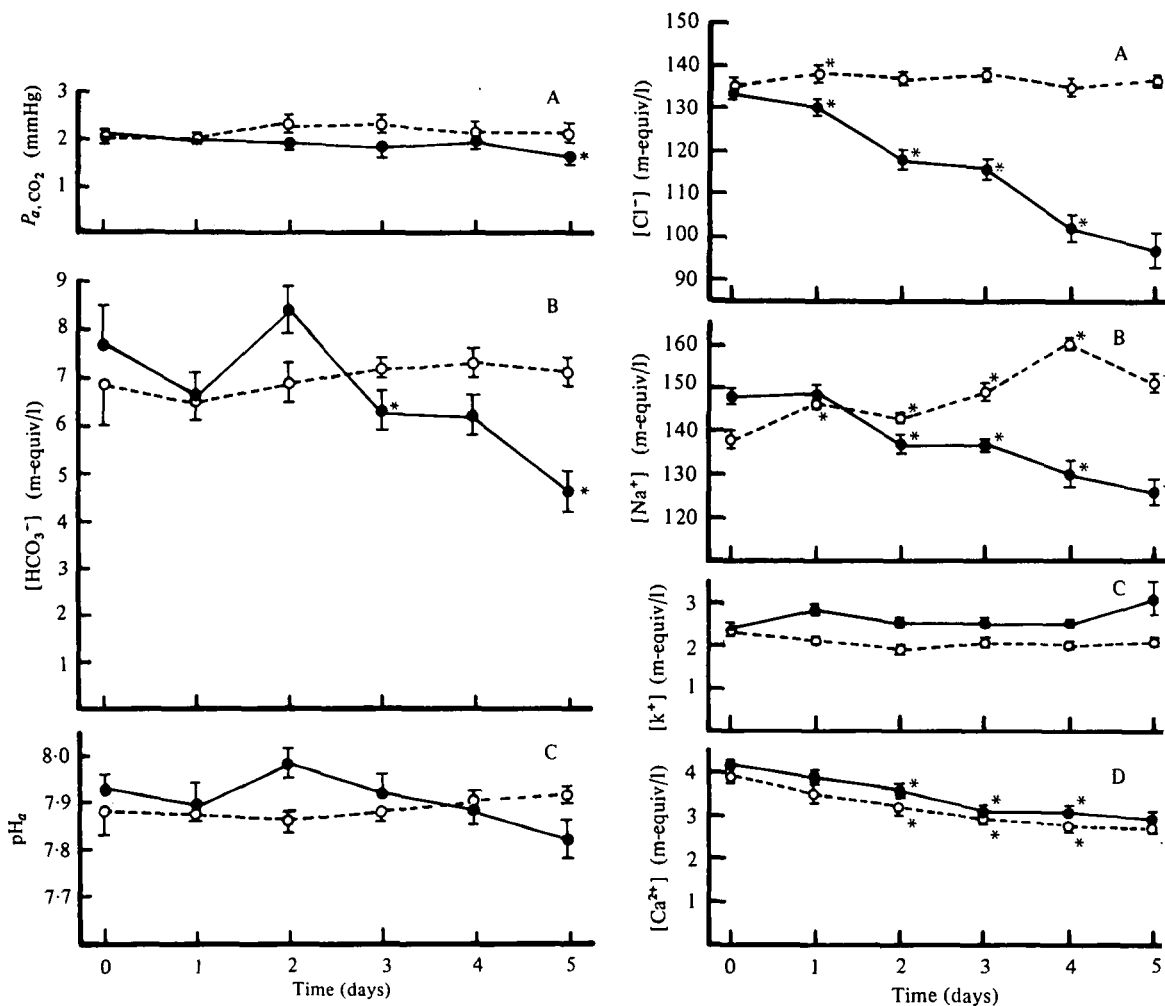
Fig. 6. Blood acid–base state (means ± one S.E.M.) in rainbow trout acutely exposed to soft water without prior acclimation. (A) Arterial CO_2 tension. (B) Arterial bicarbonate concentration. (C) Arterial pH. Animals either held at neutral pH throughout (controls, O-----O, $N = 7$) or exposed to low pH (●—●, $N = 5$) following the day o blood sample. Asterisks indicate significant difference ($P < 0.05$) from day o values (by paired t test).

Fig. 7. Plasma ion levels (means ± one S.E.M.) in rainbow trout acutely exposed to soft water without prior acclimation. Animals either held at neutral pH throughout (controls, O-----O, $N = 7$) or exposed to low pH (●—●, $N = 5$) following the day o blood sample. Asterisks indicate significant difference ($P < 0.05$) from day o values (by paired t test).

rose in controls during this period (Fig. 7B). Changes in [K$^+$] and [Ca^{2+}] (Fig. 7C, D) were similar to those seen in the acclimated soft-water group (Fig. 5C, D). Thus the ionic disturbances caused by external acid were at least as great in trout acutely exposed to soft water as in those acclimated to it for 2 weeks.

The unacclimated soft-water control trial indicated that a 2-week acclimation period was adequate. After only 5 days, all parameters except [Ca^{2+}] (Figs. 6, were identical to those seen in acclimated fish (Table 2).
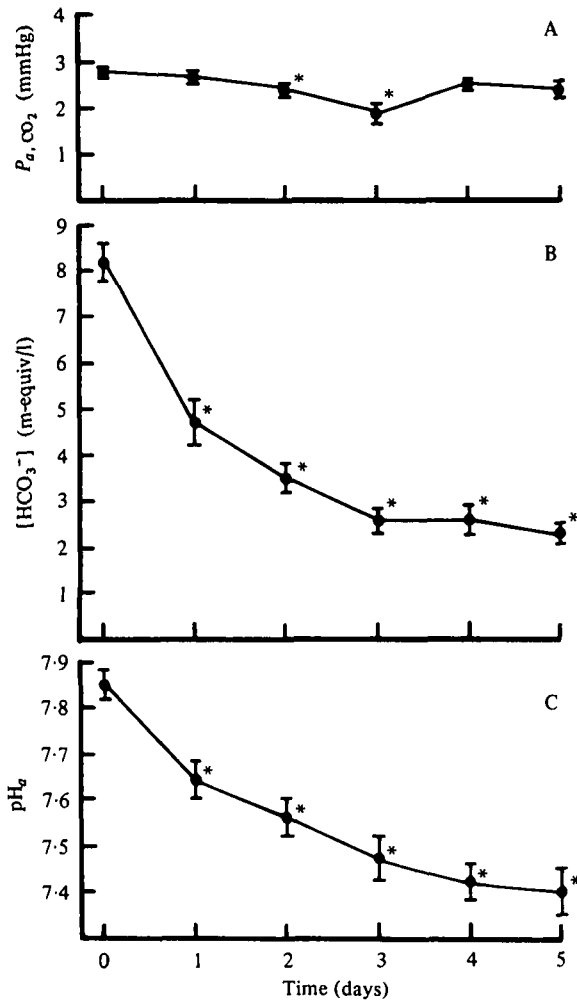
Fig. 8. Blood acid–base state (means ± one S.E.M., $N = 7$) in rainbow trout acclimated to high [Ca²⁺], low [salt] water. (A) Arterial CO₂ tension. (B) Arterial bicarbonate concentration. (C) Arterial pH. Animals were exposed to low pH following the day o blood sample. Asterisks indicate significant difference from day o values (by paired t test).

Acclimated high $[Ca^{2+}]$, low $[salt]$ water

These experiments were designed to test whether the differences in physiological effect that were observed between hard and soft acidified water were due to the difference in [Ca²⁺]. The fish were acclimated to water in which [Ca²⁺] approximated the level found in hard water, whereas other ion levels were similar to those in soft water (Table 1).

After acclimation, values for acid–base parameters resembled those in fish acclimated to hard rather than soft water (Table 2). However, plasma [Na⁺] and [Cl⁻] were significantly lower than in either group.

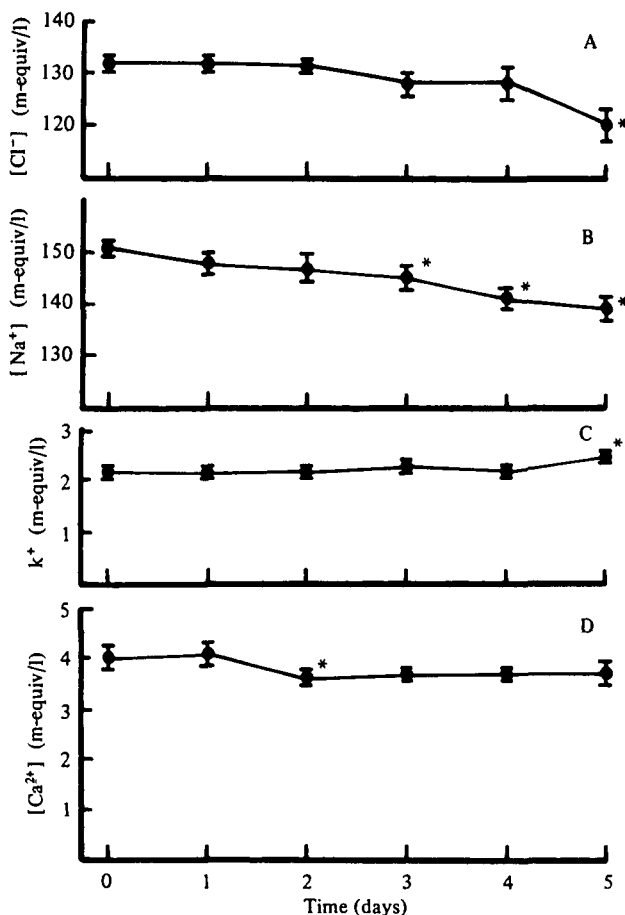The overall response to acid exposure in high [Ca²⁺], low [salt] water was very

Fig. 9. Plasma ion levels (means ± one S.E.M., $N = 7$) in rainbow trout acclimated to high [Ca^{2+}], low [salt] water. Animals were exposed to low pH following the day o blood sample. Asterisks indicate significant difference from day o values (by paired t test).

similar to that seen in hard water and very different from that seen in soft water. The fish developed a blood acid–base disturbance (Fig. 8) that was substantially larger than that seen in the soft-water experimental series (Figs. 4, 6). The pattern of acid accumulation (Fig. 2D) was very similar to that in hard water (Fig. 2A), with the bulk occurring over the first 2 days although the net loading by day 5 was higher, being 9·3 ± 0·8 m-equiv/l. The decrease in plasma [Cl⁻] (Fig. 9A) was similar to that seen in the hard water series while the decrease in [Na⁺] (Fig. 9B) was significantly less. Plasma [K⁺] and [Ca²⁺] (Fig. 9C, D) also followed the hard water pattern (Fig. 3C, D). The fact that in high [Ca²⁺], low [salt] water the acid–base disturbance was even greater and the ionic disturbance even less than in acidified hard water may be related to the higher environmental Ca²⁺ in the former (2·7 vs 1·6 m-equiv/l, Table 1).
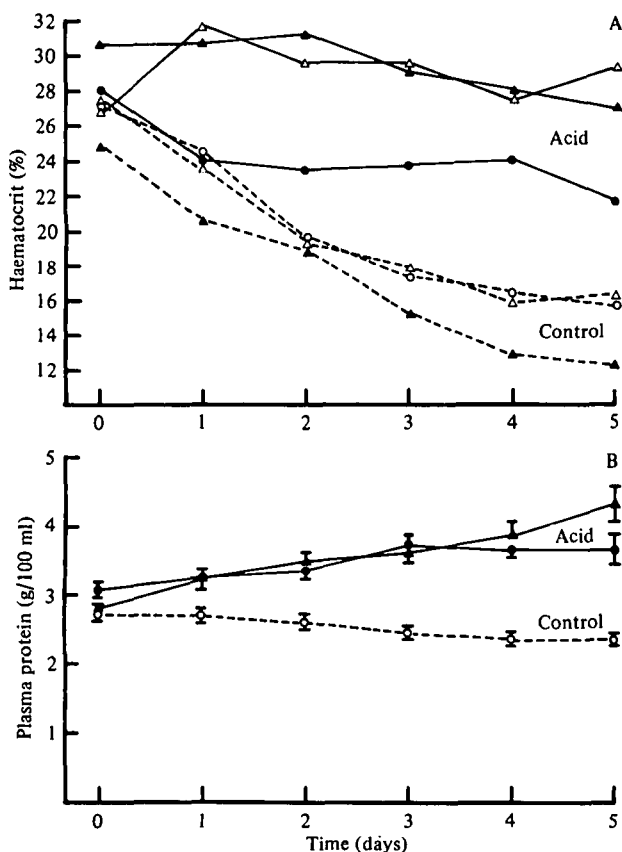
Fig. 10. Haematocrit (A; means only, S.E.M.s not drawn from the sake of clarity) and plasma protein concentration (B) in rainbow trout either held at neutral pH (-----) or exposed to low pH (———) following the day o blood sample. O, Acclimated hard water series; ▲, acclimated soft water series; △, unacclimated soft water series; ●, high [Ca²⁺], low [salt] water series.

Blood volume changes in acidified hard and soft water

Haematocrit and plasma protein concentration decreased in the control groups (Fig. 10), as might be expected as a result of repetitive blood sampling. In acid-exposed groups, however, there was substantially less decline in haematocrit (Fig. 10A), and an increase in plasma protein (Fig. 10B).

Day 5 blood volumes (BV_f) in acid-exposed fish, calculated according to haematocrit changes (see Methods), were lower than the assumed initial blood volume of 5·0 ml/ 100 g. Data from the acidified hard-water series could not be analysed (see Methods) but in the high [Ca²⁺], low [salt] series, BV_fs averaged $2·94 \pm 0·23$ ml/100 g. A similar figure for BV_f ($3·19 \pm 0·28$ ml/100 g) was obtained in the acidified soft-water series (acclimated plus unacclimated experiments). However, these BV_f estimates were quite variable within each series, probably partly because haematocrit can vary independently of blood volume. The BV_fs calculated from changes in plasma protein (see Methods) were much more consistent. In fish exposed to acid in high [Ca²⁺], low [salt] water, the average BV_f was $4·13 \pm 0·14$ ml/100 g ($N = 7$), significantly

higher than the average BV_f in acclimated soft-water fish ($3\cdot14\pm0\cdot17$ ml/100 g, $N = 7$). These estimates represent a reduction in blood volume of $17\cdot5\pm2\cdot7\%$ and $37\cdot4\pm3\cdot4\%$, respectively, from the assumed BV_i of $5\cdot0$ ml/100 g.

Acid–base and ionic disturbances associated with death at low pH

An additional eleven fish died during acid exposure. The data from these fish have been excluded from Figs. 1–10. While these fish exhibited a normal acid-base and ionic state on day 0, the physiological disturbances they exhibited during acid exposure were generally accentuated relative to the survivors. Since these disturbances may more clearly indicate the key toxic mechanism(s) the final measurements prior to death for each mortality are recorded in Table 3. In the light of the preceding results the data are grouped as deaths in either high [Ca^{2+}] (i.e., hard-water-acclimated and high [Ca^{2+}], low [salt] acclimated series) or low [Ca^{2+}] water (i.e. soft-water-acclimated and -unacclimated series). In high [Ca^{2+}] water, 3 out of 27 fish (11%) died within 6 days (one additional death was documented on Day 9). In low [Ca^{2+}] water, 8 out of 16 fish (50%) died within the same period.

Within each group the terminal data are listed in Table 3 in order of the approximate time before death at which the final sample was taken. While these data are somewhat variable, they do indicate, when compared with control values (Table 2) the major physiological disturbances accompanying death at low pH. First, the major difference in the extent of the acid–base disturbance between high and low [Ca^{2+}] water is clearly substantiated. In acidified high [Ca^{2+}] water, blood pH_as within 20 h of death were at least $0\cdot4$ unit below normal and accompanied by a substantial depression of plasma [HCO_3^-]. In low [Ca^{2+}] water on the other hand, blood acid–base balance was little affected until 2 h from death. Secondly, in all low [Ca^{2+}] animals, except fish F, plasma [Na^+] and [Cl^-] were substantially depressed (on average by 37 and 45 m-equiv/l, respectively) below acclimated soft-water control levels (Table 2). The one high [Ca^{2+}] animal in which terminal ion measurements were obtained showed a similar depression, but the data are too limited to draw any conclusions. Thirdly, in both high and low [Ca^{2+}] water, the terminal haematocrits and plasma protein concentrations (where measured) were, for the most part, substantially higher than control values. Furthermore, a trend of increasing haematocrit with increasing proximity to death was apparent. These results suggest a major and progressive loss of plasma water as death approaches. Fourthly, K^+ levels tended to rise as much as twofold above normal while Ca^{2+} levels tended to fall. Finally, there was no particular evidence of a major disturbance in gas exchange or transport. Arterial P_{CO_2} was elevated in only three fish and then only to levels roughly corresponding to normal venous levels (C. M. Wood, unpublished data). Similarly, the elevation in blood lactate which would indicated tissue hypoxia and impaired blood O_2 transport was relatively minor (cf. Kobayashi & Wood, 1980) in those animals in which this parameter was measured.

Table 3. *Terminal measurements in fish succumbing to low pH exposure*

Fish	Day of death	Hours before death*	Haematocrit (%)	pH$_a$	P$_{a,CO_2}$ (torr)	HCO$_3^-$	Cl$^-$	Na$^+$	K$^+$	Ca^{2+}	Lactate	Plasma protein (g/100 ml)
								(m-equiv/l)				
High [Ca^{2+}] water												
A	9	20	29·5	7·30	4·7	3·44	—	—	—	—	—	—
B	4	20	38·0	7·40	2·6	2·41	—	—	—	—	—	—
C	4	15	22·4	7·49	2·0	2·42	79·0	92·8	4·7	1·2	—	—
D	3	4	36·8	7·41	2·1	2·05	—	—	—	—	—	—
$\bar{X} \pm 1$ S.E.M.	—	—	31·7 ±3·6	7·40 ±0·04	2·9 ±0·6	2·58 ±0·30						
Low [Ca^{2+}] water												
E	5	20	19·7	7·78	2·1	5·32	87·0	108·5	3·3	2·9	1·6	4·5
F	1	19	24·2	7·97	1·5	5·99	127·0	145·0	2·4	3·6	—	—
G	4	11	39·7	7·79	4·2	10·89	88·0	111·1	3·7	2·8	3·3	3·7
H	5	8	36·4	7·72	1·6	3·53	89·0	119·9	4·6	4·1	4·2	5·2
I	2	8	38·9	7·80	1·1	2·97	99·0	124·0	2·3	4·2	—	4·4
J	5	6	30·6	7·80	1·6	4·26	84·0	103·3	3·3	2·6	1·4	5·0
K	5	2	32·7	7·60	1·9	3·09	76·0	100·6	3·5	3·3	3·7	—
L	3	At death	62·1	7·37	4·0	—	88·0	123·4	3·4	4·5	—	4·5
$\bar{X} \pm 1$ S.E.M.	—		35·5 ±4·5	7·73 ±0·06	2·3 ±0·4	5·15 ±1·05	92·3 ±5·4	117·0 ±5·1	3·3 ±0·3	3·5 ±0·2	2·8 ±0·6	4·5 ±0·3

* These are the times between when the last blood sample was taken and when death was first noted. Thus they are maximum estimates and may, in some fish, be in error by up to 10 h.
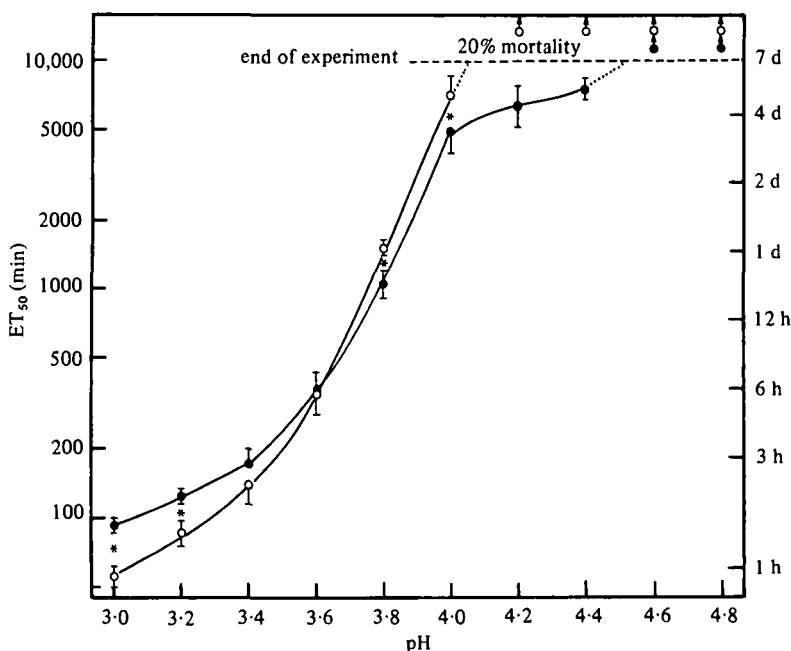
Fig. 11. Relationship between median lethal resistance time (ET_{50}) and environmental pH in hard ($[Ca^{2+}] = 3\cdot3$ m-equiv/l; ●) and soft ($[Ca^{2+}] = 0\cdot2$ m-equiv/l; ○) water. Asterisks indicate significant difference ($P < 0\cdot05$) between hard and soft water ET_{50}s at the same pH. Except where noted no mortalities occurred in 7 days from tests at pH $\geqslant 4\cdot2$ in soft water and pH $\geqslant 4\cdot6$ in hard water. Vertical bars indicate 95 % confidence limits.

Toxicity tests

At acutely toxic environmental pH levels ($3\cdot0$, $3\cdot2$), median lethal resistance times for fingerling trout were significantly greater in hard water ($[Ca^{2+}] = 3\cdot3$ m-equiv/l) than in soft water ($[Ca^{2+}] = 0\cdot2$ m-equiv/l) (Fig. 11). At slightly higher pH levels ($3\cdot4$, $3\cdot6$) the toxicity curves coincided, while at still higher levels the situation was reversed. Trout survived significantly longer in soft water than in hard water at pH $3\cdot8$ and $4\cdot0$. The same was true at pH $4\cdot2$ and $4\cdot4$, though statistical tests could not be performed because the majority of the soft-water animals survived beyond the end of the 7-day experimental period. This should not be interpreted as indefinite survival; indeed the results provide no indication that a true incipient lethal threshold (Sprague, 1969) was defined within the 7-day period.

There were no consistent differences between hard and soft water trials at comparable pH levels in the slope functions of the log time vs probit mortality curves (Litchfield, 1949) from which the data in Fig. 11 are derived. However, within each water type, the slope functions increased progressively and significantly with pH, from about $1\cdot1$ at pH $3\cdot0$ to $1\cdot4$ at pH $4\cdot0$.

DISCUSSION

The physiological responses to acid exposure

At a mean pH of 4·3, the physiological disturbances in the rainbow trout are clearly dependent on the level of calcium in the environment, and not the levels of other ions (at least over the ranges tested here). High $[Ca^{2+}]$ was associated with a marked blood acidosis and a relatively small plasma ionic disturbance as found previously in trout (Neville, 1979) and carp (N. Heisler, personal communication). The reverse was found with low $[Ca^{2+}]$. The acclimated soft-water series ($[Ca^{2+}] =$ 0·3 m-equiv/l), the acclimated hard water series ($[Ca^{2+}] = 1·6$ m-equiv/l) and the acclimated high $[Ca^{2+}]$, low [salt] water series ($[Ca^{2+}] = 2·7$ m-equiv/l) form a sequence of increasing calcium levels in which this phenomenon is well illustrated (Figs. 1, 2, 3, 4, 5, 8, 9). Low $[Ca^{2+}]$ also seemed to be associated with a greater reduction in blood volume during acid exposure. Clearly, these results indicate that the effect of low external pH on ion regulation in fish cannot be considered in isolation from its effect on acid–base regulation, and neither effect can be considered in isolation from the influence of external $[Ca^{2+}]$. Such interrelations are to be expected, since it is known that ionoregulation and acid–base regulation are coupled to some degree by electroneutral exchanges at the gills (Na^+ vs, H^+ or NH_4^+; Kerstetter, Kirschner & Rafuse, 1970; Maetz, 1973; Payan, 1978 and Cl^- vs HCO_3^- or OH^-; Kerstetter & Kirschner, 1972; DeRenzis & Maetz, 1973; DeRenzis, 1975) and that Ca^{2+} is an important modulator of branchial ion and water permeability in fish (Isaia & Masoni, 1976).

A number of studies have shown that exposure of fish to acidic external pH (Ca^{2+} level unspecified; Packer & Dunson, 1970, 1972; Maetz, 1973; 1·0 m-equiv/l Ca^{2+}; McWilliams & Potts, 1978) results in an inhibition of active Na^+ influx and a stimulation of its diffusional efflux, leading to net Na^+ loss and reduction of body Na^+ levels. Reductions in plasma $[Na^+]$ occurred in all acid-exposed groups in the present study (Figs. 3B, 5B, 7B, 9B). The smaller reduction in plasma $[Cl^-]$ than $[Na^+]$ in the trout at pH 4·3 in high $[Ca^{2+}]$ water (Figs. 3, 9) suggests that Cl diffusional losses and/or active transport were less affected than Na^+ fluxes, at least under high $[Ca^{2+}]$ conditions. Chloride fluxes under acidic conditions have not previously been examined to a similar extent but Maetz (1973) showed that Cl^- net flux was not affected by a small reduction in external pH (from 7·2 to 6·1) whereas Na^+ influx and net flux were significantly reduced.

A reduction in environmental $[Ca^{2+}]$ has been shown to increase the passive efflux from fish of both Na^+ (Potts & Fleming, 1971; Cuthbert & Maetz, 1972; Eddy, 1975) and Cl^- (Eddy, 1975). In solutions near neutrality the increased effluxes were compensated by increased influxes so that no net loss of either ion occurred (Cuthbert & Maetz, 1972; Eddy, 1975). This probably explains the observation (Table 2) that plasma Na^+ and Cl^- levels were initially reduced by acute soft-water exposure but were entirely compensated after 2 weeks acclimation. The more extensive losses of both plasma Na^+ and Cl^- which occurred with acid exposure in low $[Ca^{2+}]$ water (Fig. 5) relative to high $[Ca^{2+}]$ water (Figs. 3, 9) must reflect a greater imbalance between influxes and effluxes.

In addition to raising diffusive salt losses, low $[Ca^{2+}]$ can be expected to increase

the passive penetration of protons at the gills during acid exposure (McWilliams Potts, 1978). However, the blood acidosis accompanying low external pH was negligible in low [Ca^{2+}] relative to high [Ca^{2+}] (Figs. 1, 2, 4). This suggests that a major elevation of proton excretion took place during acid exposure in low [Ca^{2+}] water. This could be linked to Na^+ uptake (see above) which is elevated in low [Ca^{2+}] water (Cuthbert & Maetz, 1972; Eddy, 1975). An elevation of Na^+/H^+ exchange could be the reason for the increase in plasma Na^+ level observed in the unacclimated soft-water controls (Fig. 7) and for the metabolic alkalosis observed on day 2 in the acid-exposed group (Fig. 6).

Implicit in the above arguments are three conditions. First, activation of Na^+ transport by low [Ca^{2+}] (Eddy, 1975) must predominate over the inhibition of Na^+ transport by low pH (Packer & Dunson, 1970; Maetz, 1973; McWilliams & Potts, 1978). Secondly, the stimulation of Na^+ efflux by low pH (Packer & Dunson, 1970; McWilliams & Potts, 1978) must be greater than the stimulation of Na^+ influx by low [Ca^{2+}] to explain the large depression in plasma Na^+ levels (Figs. 5B, 7B). Thirdly, any activation of Cl^-/base exchange at low Ca^{2+} levels and acid pH must be less than that of Na^+/proton exchange. The latter appears to be the case during the correction of an acidosis associated with salt depletion in goldfish (DeRenzis & Maetz, 1973). It is also supported by the significantly greater depression of plasma [Cl^-] by day 5 of acid exposure in both low [Ca^{2+}] series (Figs. 5, 7).

In addition to the above mechanisms it seems highly probable that kidney function will also play a significant role in the overall compensation to acid stress (Wood & Caldwell, 1978; Kobayashi & Wood, 1980). Clearly, direct and simultaneous measurements of branchial and renal ion and acid fluxes together with continuous assessment of blood acid–base and electrolyte status will be required to fully identify the complex interactions between water [Ca^{2+}], water pH, ionoregulation and acid–base regulation. Nevertheless, the present findings do establish the critical importance of environmental [Ca^{2+}], usually the major component of water hardness, in determining the nature of the response to acid exposure.

Mechanisms of acid toxicity

As Sprague (1971) has pointed out, any environmental stress, such as low pH, will produce a host of physiological disturbances, but most will be within the range of adaptation of the animal and therefore of little influence on individual survival. If the effects are to be used for predictive purposes (e.g. sublethal bioassays, field surveys) it will be necessary to understand which effects cause death, under a variety of conditions.

At critically low pH levels (< 4.0) where mortality is 100% and death occurs within hours rather than days, a failure of O_2 delivery to the tissues is probably of primary importance. A pronounced accumulation of mucus on the gills (Plonka & Neff, 1969; Daye & Garside, 1976; Ultsch & Gros, 1979) and a sloughing of gill epithelial tissue (Daye & Garside, 1976) may severely impair branchial O_2 diffusion. This, combined with a marked reduction in blood O_2 capacity (Root effect), due to massive acidosis (Packer & Dunson, 1970, 1972; Packer, 1979; N. Heisler, personal communication), results in eventual cellular anoxia. However, such pH levels are rarely encountered by fish in the wild. Much more common will be chronic expos

pHs in the range 4·5–6·0 which occurs when poorly buffered soft-water lakes are gradually titrated to the endpoint ($\sim$ 4·5) of the HCO_3^-/CO_2 buffer curve by long-term acid input. This may be accompanied by episodic excursions down to $\sim$ pH 4·0 during rainstorms and snow melt (Leivestad *et al.* 1976; Harvey, 1979).

The present results clearly show that O_2 delivery failure is not the cause of death at these more moderate pH levels. Mucus accumulation on the gills was not seen and blood P_{CO_2} levels remained normal. Blood lactate levels were either unaffected (hard water; Fig. 1 D) or marginally elevated (soft water; Fig. 4 D) after 5 days exposure. Terminal measurements on dying fish (Table 3) were well within the normal range of variation (Kobayashi & Wood, 1980; C. M. Wood, unpublished results). These findings would also seem to rule out possible circulatory failure associated with reductions in blood volume.

In those few fish which succumbed in high $[Ca^{2+}]$ water in the present study, the terminal blood acid–base disturbance was slightly more severe than in the survivors (Table 3; Figs. 1, 9). The extent of this pH depression (about 0·4 units) is within the short-term tolerance of the trout (Holeton & Randall, 1967; Kiceniuk & Jones, 1977; Kobayashi & Wood, 1980) but chronic acidosis of this magnitude may be more serious, particularly if combined with an ionic disturbance. A severe ionic disturbance was noted in one fish that died at pH 4·3 in high $[Ca^{2+}]$. Although these data are too limited to be conclusive we have recently noted (D. G. McDonald & C. M. Wood, unpublished results) similar ionic disturbances associated with deaths in hard water acidified with H_2SO_4.

In low Ca^{2+} water the results (Table 3) more clearly suggest that ionoregulatory failure is the sole toxic mechanism of low pH; a conclusion supported by field studies (Leivestad & Muniz, 1976; Leivestad *et al.* 1976). In the present study, percentage mortality was markedly increased relative to high $[Ca^{2+}]$ water at the same acidic pH (50% vs 11% over 6 days). In both surviving and dying fish, blood acid–base status was little affected (Figs. 4, 6; Table 3) but plasma ion levels were severely disturbed (Figs. 5, 7; Table 3). Prior to death (Table 3), plasma Na^+ and Cl^- concentrations were uniformly depressed by about 25% and 30% respectively while $[K^+]$ showed an almost 1·6-fold increase.

Toxicity trials with fingerling trout were performed to determine whether these same differences in acid toxicity and toxic mechanism could also be detected by the classical lethality bioassay. In these tests, differences in toxicity are detected as differences in median lethal resistance times while changes in the slope functions of log time vs probit mortality relationships are interpreted as changes in toxic mechanisms (Sprague, 1969). Progressive changes in slope function were found with increasing pH in both hard and soft water, which tends to confirm that a gradual transition from one key toxic mechanism (e.g. oxygen delivery failure) to another (e.g. acid–base and/or ion regulatory failure) may have occurred. However, there were no consistent differences in the slope functions between hard and soft water indicative of a difference in modes of toxicity between the two environments. Furthermore, while $[Ca^{2+}]$ prolonged survival at acutely toxic pH levels (3·0, 3·2) the reverse occurred at more moderate levels (3·8–4·4). Very similar data were presented by Lloyd & Jordan (1964) in their comparison of an even wider range of calcium levels than that examined here. Although a number of factors (variations

in age, size, degree of restraint, metabolic rate, etc.) may have contributed to t█ differences between the toxicological and physiological studies, the fact that they gave fundamentally different results emphasizes that extreme caution must be used in extrapolating from one type of study to the other. Nevertheless, there is some common ground in so far as both methods indicate the critical importance of environmental calcium levels. Relatively minor variations in water $[Ca^{2+}]$ may thus spell the difference between survival and eventual extinction for fish populations under acid stress in the wild.

REFERENCES

BEAMISH, R. J., LOCKHART, W. L., VAN LOON, J. C. & HARVEY, H. H. (1975). Long-term acidification of a lake and resulting effects on fishes. *Ambio* 4, 98–102.

COGBILL, C. V. & LIKENS, G. E. (1974). Acid precipitation in the north-eastern United States. *Wat. Resour. Res.* 10, 1133–1137.

CUTHBERT, A. W. & MAETZ, J. (1972). The effects of calcium and magnesium on sodium fluxes through gills of *Carassius auratus*. *J. Physiol., Lond.* 221, 633–643.

DAYE, P. G. & GARSIDE, E. T. (1976). Histopathologic changes in superficial tissues of brook trout, *Salvelinus fontinalis* (Mitchell), exposed to acute and chronic levels of pH. *Can. J. Zool.* 54, 2140–2155.

DERENZIS, G. (1975). The branchial chloride pump in the goldfish *Carassius auratus*: relationship between Cl^-/HCO_3^- and Cl^-/Cl^- exchanges and the effect of thiocyanate. *J. exp. Biol.* 63, 587–602.

DERENZIS, G. & MAETZ, J. (1973). Studies on the mechanism of chloride absorption by the goldfish gill. Relation with acid–base regulation. *J. exp. Biol.* 59, 339–358.

DILLON, P. J., JEFFRIES, D. S., SNYDER, W., REID, R., YAN, N. D., EVANS, D., MOSS, J. & SCHNEIDER, W. A. (1978). Acidic precipitation in south-central Ontario: recent observations. *J. Fish. Res. Bd Can.* 35, 809–815.

EDDY, F. B. (1975). The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, *Carassius auratus*. *J. comp. Physiol.* 96, 131–142.

HARVEY, H. H. (1975). Fish populations in a large group of acid-stressed lakes. *Verh. Internat. Verein. Limnol.* 19, 2406–2417.

HARVEY, H. H. (1979). The acid deposition problem and emerging research needs in the toxicology of fishes. *Proc. Fifth Annual Aquatic Toxicity Workshop*, Hamilton, Ontario, Nov. 7–9, 1978. *Fish. Mar. Serv. Tech. Rep.* no. 862, 115–128.

INTERNATIONAL JOINT COMMISSION (1976). *Report of the Water Quality Objectives Subcommittee.* Regional Office of the IJC, Windsor, Ontario, Canada.

HOLETON, G. F. & RANDALL, D. J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood afferent and efferent to the gills of rainbow trout. *J. exp. Biol.* 46, 317–327.

ISAIA, J. & MASONI, A. (1976). The effects of calcium and magnesium on water and ionic permeabilities in the sea water adapted eel, *Anguilla anguilla* L. *J. comp. Physiol.* 109, 221–233.

JENSON, K. W. & SNEKVIK, E. (1972). Low pH levels wipe out salmon and trout populations in southernmost Norway. *Ambio* 1, 223–225.

KERSTETTER, T. H. & KIRSCHNER, L. B. (1972). Active chloride transport by the gills of rainbow trout *Salmo gairdneri*. *J. exp. Biol.* 56, 263–272.

KERSTETTER, T. H., KIRSCHNER, L. B. & RAFUSE, D. D. (1970). On the mechanism of sodium ion transport by the irrigated gills of rainbow trout. *Salmo gairdneri*. *J. gen. Physiol.* 56, 342–359.

KICENIUK, J. W. & JONES, D. R. (1977). The oxygen transport system in trout, *Salmo gairdneri* dur█ sustained exercise. *J. exp. Biol.* 69, 247–260.

KOBAYASHI, K. & WOOD, C. M. (1980). The response of the kidney of the freshwater rainbow trout to true metabolic acidosis. *J. exp. Biol.* **84**, 227–244.

LEIVESTAD, H., HENDREY, G., MUNIZ, I. P. & SNEKVIK, E. (1976). Effect of acid precipitation on freshwater organisms. In *Impact of Acid Precipitation on Forest and Freshwater Ecosystems in Norway* (ed. F. H. Braekke). (SNSF-project Research Report FR 6/76, Aas-NLH, Norway, 1976), pp. 86–111.

LEIVESTAD, H. & MUNIZ, I. P. (1976). Fish kill at low pH in a Norwegian river. *Nature, Lond.* **259**, 391–392.

LITCHFIELD, J. T. (1949). A method for rapid graphic solution of time–percent effect curves. *J. Pharmac. exp. Ther.* **97**, 399–408.

LLOYD, R. & JORDAN, D. H. M. (1964). Some factors affecting the resistance of rainbow trout (*Salmo gairdneri*) to acid waters. *Int. J. Air. Wat. Pollut.* **8**, 393–403.

MAETZ, J. (1973). Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus. J. exp. Biol.* **58**, 255–275.

MCDONALD, D. G., BOUTILIER, R. G. & TOEWS, D. P. (1980). The effects of enforced activity on ventilation, circulation and blood acid–base balance in the semi-terrestrial anuran, *Bufo marinus. J. exp. Biol.* **84**, 273–287.

MCWILLIAMS, P. G. & POTTS, W. T. W. (1978). The effects of pH and calcium concentrations on gill potentials in the brown trout, *Salmo trutta. J. comp. Physiol.* **126**, 277–286.

MUDGE, J. E. & NEFF, W. H. (1971). Sodium and potassium levels in serum of acid-exposed brook trout, *Salvelinus fontinalis. Proc. Pa Acad. Sci.* **45**, 101–103.

NEVILLE, C. M. (1979). Sublethal effects of environmental acidification on rainbow trout, *Salmo gairdneri. J. Fish. Res. Can.* **36**, 84–87.

ODÉN, S. (1976). The acidity problem – an outline of concepts. *Water Air & Soil Pollut.* **6**, 137–166.

PACKER, R. K. (1979). Acid-base balance and gas exchange in brook trout (*Salvelinus fontinalis*) exposed to acidic environments. *J. exp. Biol.* **79**, 127–134.

PACKER, R. K. & DUNSON, W. A. (1970). Effects of low environmental pH on blood pH and sodium balance of brook trout. *J. exp. Zool.* **174**, 65–72.

PACKER, R. K. & DUNSON, W. A. (1972). Anoxia and sodium loss associated with the death of brook trout at low pH. *Comp. Biochem. Physiol.* **41 A**, 17–26.

PAYAN, P. (1978). A study of the Na^+/NH_4^+ exchange across the gill of the perfused head of the trout, *Salmo gairdneri. J. comp. Physiol.* **124**, 181–188.

PLONKA, A. C. & NEFF, W. H. (1969). Mucopolysaccharide histochemistry of gill epithelial secretions in brook trout exposed to acid pH. *Proc. Pa Acad. Sci.* **43**, 53–55.

POTTS, W. T. W. & FLEMING, W. R. (1971). The effect of environmental calcium and ovine prolactin on sodium balance in *Fundulus kansae. J. exp. Biol.* **55**, 63–76.

ROCH, M. & MALY, E. J. (1979). Relationship of cadmium-induced hypocalcemia with mortality in rainbow trout, *Salmo gairdneri* and the influence of temperature on toxicity. *J. Fish. Res. Can.* **36**, 1297–1303.

SCHOFIELD, C. L. (1976). Acid precipitation: effects on fish. *Ambio* **5**, 228–230.

SEVERINGHAUS, J. W. (1965). *Handbook of Physiology*, Section 3, Respiration, vol. 2, 1480.

SIGMA (1976). *The Quantitative Determination of Pyruvic Acid and Lactic Acid in Whole Blood*. Sigma Technical Bulletin No. 826 U.V. Sigma Chemical Company, St Louis Missouri.

SMITH, L. S. & BELL, G. R. (1964). A technique for prolonged blood sampling in free swimming salmon. *J. Fish. Res. Bd Can.* **21**, 711–717.

SPRAGUE, J. B. (1969). Measurement of pollutant toxicity of fish. I. Bioassay methods for acute toxicity. *Water Resour.* **3**, 793–821.

SPRAGUE, J. B. (1971). Measurement of pollution toxicity to Fish. III. Sublethal effects and 'safe' concentrations. *Water. Resour.* **5**, 245–266.

STEVENS, E. D. (1968). The effect of exercise on the distribution of blood to various organs in rainbow trout. *Comp. Biochem. Physiol.* **25**, 615–625.

ULTSCH, G. R. & GROS, G. (1979). Mucus as a diffusion barrier to oxygen: possible role in O_2 uptake at low pH in carp (*Cyprinus carpio*) gills. *Comp. Biochem. Physiol.* **62 A**, 685–689.

VAALA, S. S. & MITCHELL, R. B. (1970). Blood oxygen tension changes in acid exposed brook trout. *Proc. Pa Acad. Sci.* **44**, 41–44.

WOLF, K. (1963). Physiological salines for freshwater teleosts. *Prog. Fish. Cult.* **25**, 135–140.

WOOD, C. M. & CALDWELL, F. H. (1978). Renal regulation of acid–base balance in a freshwater fish. *J. exp. Zool.* **205**, 301–307.

WOOD, C. M., MCMAHON, B. R. & MCDONALD, D. G. (1977). An analysis of changes in blood pH following exhausting activity in the starry flounder, *Platichthys stellatus. J. exp. Biol.* **69**. 173–185.