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THE INTERACTION OF ENVIRONMENTAL CALCIUM AND LOW pH ON THE PHYSIOLOGY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

I. BRANCHIAL AND RENAL NET ION AND H^+ FLUXES

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SUMMARY

Exposure of adult rainbow trout to low pH (pH 4·3) in soft water (Ca^{2+} = 223 μequiv/l) caused a substantial ionic disturbance which arose primarily because of large net losses at the gills. In contrast, renal ion losses were low initially and declined even further because of a pronounced reduction in urine flow. A net influx of H^+ occurred across the gills but this was not sufficient to cause a blood acid-base disturbance or a renal response. Although branchial ion and H^+ fluxes declined with time, blood ion levels did not return to normal and many of the fish died. Further reduction in water calcium (Ca^{2+} = 69 μequiv/l) provoked a higher mortality and a more substantial ionic imbalance. These results contrast sharply with the effects on trout of acid exposure in hard water ($Ca^{2+} \geq 1600$ μequiv/l), where net ion losses and mortality are reduced and H^+ uptake increased. A preliminary model for the interaction of low pH and calcium is proposed and evidence for adaptation to acid stress and for the origin of acid lethality is discussed.

INTRODUCTION

Atmospheric sulphur dioxide emission has been responsible for the acidification of lakes and the depletion of fish stocks in many parts of the world. Loss of fish populations has been attributed (Schofield, 1976; Leivestad, Hendry, Muniz & Snekvik, 1976; Harvey, 1979; Fromm, 1980) to spawning failure and diminished hatching success at moderately acid pH levels (pH < 6·0) and to fish kills during toxic pH excursions (pH < 4·5), such as would occur during snow melt. One of the most important aspects of this phenomenon is the finding that acid stress is apparently more severe in waters of very low ionic strength. Leivestad *et al.* 1976, in a survey of 941 lakes in southern Norway, found that the incidence of barren lakes increased not only with decreasing pH but also with decreasing conductivity. A more recent survey by Wright & Snekvik (1978) indicated that calcium was the most important ion determining fisheries status, more important, in fact, than pH. This property of environmental Ca^{2+} has now been confirmed in several laboratory studies (Leivestad *et al.* 1976; Trojnar, 1977; Carrick, 1979; Brown, 1981; Graham & Wood, 1981). Brown (1981),

Key words: Low pH, calcium, trout.

for example, showed that elevating Ca^{2+} reduced the toxicity of low pH (pH' 3·5–4·0) to brown trout fingerlings by a greater extent than any other ion tested (Na^+, K^+, Mg^{2+} and Al^{3+}).

Initial studies of the physiological basis of this phenomenon (McDonald, Hōbe & Wood, 1980) emphasized the important modulating effect of Ca^{2+} on acid-base and ionoregulatory mechanisms. Exposure of rainbow trout to low pH (pH 4·3) in soft water (Ca^{2+} = 300 μequiv/l) led to a more pronounced plasma ionic disturbance and to a higher mortality but only to a minor acid-base disturbance compared to that developing at the same pH in moderately hard water (Ca^{2+} = 1600 μequiv/l). A later study (McDonald & Wood, 1981) established that the plasma ion and acid-base disturbances arose mainly at the gills through net ion losses and a net H^+ uptake. It was also shown that a substantial increase in renal acid excretion occurred in response to the appearance of a plasma acidosis. These results, however, were obtained at low pH in hard water and thus are difficult to extrapolate to acidified soft water given the qualitatively different responses of fish to the two environments (McDonald *et al.* 1980).

Thus, the purpose of the present study is to examine the branchial and renal responses of trout exposed to low pH in low [Ca^{2+}] water. This study was designed to be directly comparable to our previous study (McDonald & Wood, 1981) and therefore employs adult trout, surgically fitted with indwelling catheters for blood sampling and urine collection, and exposed to acid water (pH ≃ 4·3) in low volume, thermostatted, recirculating systems. The only major methodological difference, other than lower Ca^{2+} levels, was that branchial net ion and acid fluxes were measured on individual fish in the present study. In our previous study those fluxes were determined as a single average value on groups consisting of either six or eight fish.

METHODS

Experimental animals

Adult rainbow trout, *Salmo gairdneri* Richardson, of both sexes were obtained from hatchery stock (Spring Valley Trout Farms, Petersberg, Ontario) and held prior to experimentation in large polyethylene tanks continuously supplied with well aerated, dechlorinated tap water. Trout were fed *ad libitum* with commercial trout pellets while in the holding facilities. Prior to use, trout (182–390 g) were acclimated for 10–12 days to water of the temperature (16 ± 1 °C) and ion composition of that employed in subsequent experiments (see Table 1). This acclimation period was required because of the plasma ionic disturbances that result from acute low Ca^{2+} exposure at circumneutral pH (McDonald *et al.* 1980). Trout were starved during this period to remove the influence of diet on renal acid output (Wood & Caldwell, 1978).

Test conditions

All water for acclimation and experimentation was prepared by dilution of tap water with deionized water to give nominal Ca^{2+} levels of either 230 μequiv/l (experimental series 1) or 60 μequiv/l (series 2). The mixtures were then supplemented with NaCl and KCl to raise Na^+ and Cl^- concentrations to detectable levels for ion flux

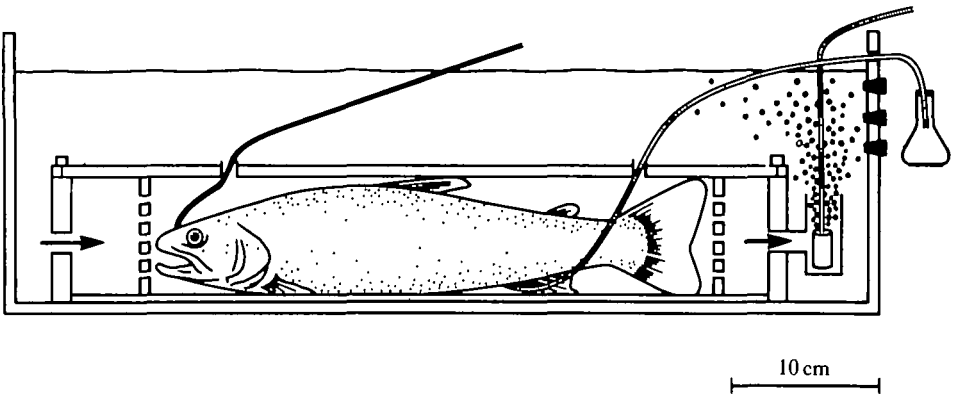

Fig. 1. Apparatus used for measurement of branchial ion and acid fluxes in 100–400 g rainbow trout. Trout fitted with catheters in the dorsal aorta and in the urinary bladder. Inner 'fish' chamber dimensions are 10 × 19 × 48 cm (width × height × length); outer 'flux' chamber, 13 × 20 × 60 cm. Water flow (~0·5 l/min), aeration and mixing was maintained by the 'air-lift' pump fitted on the rear of the fish chamber. During flux determinations, the flux chamber was covered to prevent moisture loss. Drainage plugs on the rear of the flux chamber were used to adjust volume.

determinations (Table 1). Acid water for experimental use was prepared by titration to a pH of 4·0 with H_2SO_4 and then aeration, prior to use, to remove CO_2.

For all experiments fish were confined in 2 l 'fish' chambers which were normally placed within 'flux' chambers of slightly larger dimensions (Fig. 1). The fish chambers were fitted with an air-lift pump (see Fig. 1) which circulated water through the chamber at about 0·5 l/min and also provided aeration. This design enabled the transfer of fish chambers with virtually no disturbance to the confined animal, a necessity because of the well known effects of handling stress on branchial ion fluxes (Maetz, 1974; Cameron, 1976). While fish were recovering from surgery and in the interim between flux periods the flux chambers were gravity fed from 200 l recirculated reservoirs maintained at 16 ± 1 °C. Reservoir water was changed daily to minimize accumulation of ammonia and other wastes.

For the determination of branchial fluxes, the flow into the chambers was shut off and the total volume for each fish adjusted by drainage to either 11 l (series 1) or 5 l (series 2). Temperature control during this time was maintained by bathing the flux chambers in cooling water. In series 1 experiments, the flux periods were 10 h in

Table 1. *Ion composition (means ± one S.E.M.) and pH (means, range) of test water*

T = 16 ± 1 °C. All ions in μequiv/l. High Ca^{2+} data from McDonald & Wood (1981).

	Circumneutral Series 1	Acid exposure Series 1	Acid exposure Series 2	Acid exposure High Ca^{2+}
pH	7·4 (7·2–7·7)	4·3 (4·0–5·1)	4·2 (4·1–4·5)	4·2 (3·7–5·1)
Ca^{2+}	243 (± 31)	223 (± 7)	69 (± 2)	1930 (± 30)
Na^+	245 (± 40)	315 (± 52)	311 (± 25)	860 (± 30)
Cl^-	357 (± 10)	492 (± 71)	386 (± 37)	860 (± 20)
K^+	65 (± 7)	37 (± 8)	170 (± 10)	730 (± 100)
ammonia	233 (± 31)	92 (± 6)	70 (± 7)	717 (± 103)

duration separated by 2 h intervals while chambers were flushed with either circumneutral or acid water. In series 2, the circumneutral and acid flux periods were each 4 h in duration.

Experimental protocol

Series 1

This series closely followed procedures outlined in McDonald & Wood (1981). Trout ($N = 13$) were surgically fitted under MS-222 anaesthesia with catheters in the dorsal aorta for repetitive blood sampling and in the urinary bladder for continuous urine collection. Animals were allowed 36–48 h to recover from this procedure. The experimental period consisted of 24 h at a circumneutral pH followed by 96 h of acid exposure (Table 1). During this period blood (0·6 ml) was sampled at 24 h intervals and analysed for acid-base and ion parameters. The volume removed at each sample was replaced with an equal volume of saline. Urine was collected into covered vials over 12 h intervals and analysed for ions, ammonia and titratable acidity. Water samples were collected at the beginning and end of each flux period and analysed for ions, ammonia and titratable alkalinity. The starting pH for the flux periods during acid exposure was $4{\cdot}0 \pm 0{\cdot}01$. (±one S.E.M.) Over the 10 h flux period, water pH gradually increased since no further acid was added during this time. The average increase in water pH was 1·1 units, thus the mean exposure pH, by log transformation, was 4·27 (+0·03, −0·02).

Series 2

This series examined the physiological effects of low pH in extreme soft water ($Ca^{2+} = 69\ \mu$equiv/l, Table 1). In this series, trout ($N = 12$) were fitted with dorsal aorta catheters only and were exposed to low pH for 48 h. Blood (0·8 ml) was sampled once under circumneutral pH conditions and twice during acid exposure, at +40 h and +44 h. These samples were analysed as above and also for osmolarity, α-amino nitrogen, ammonia, glucose and plasma protein. Two 4 h flux periods were conducted, the first was at circumneutral pH and the second started at +40 h of acid exposure. During these periods water samples for analysis were collected at hourly intervals.

Analytical techniques

Blood samples were collected anaerobically into chilled syringes and analysed immediately for pH, total CO_2 (whole blood and plasma) and haematocrit by methods described previously (McDonald *et al.* 1980). From these measurements, $P_{a,\,CO_2}$ and HCO_3^- were calculated according to the Henderson-Hasselbalch equation. Fresh plasma was analysed without dilution for osmolarity (Wescor vapour pressure osmometer), chloride (Radiometer CMT-10 chloride titrator) and plasma protein concentration (American Optical Goldberg refractometer), and, by dilution, for cations (Na^+, K^+ and Ca^{2+}; EEL and Coleman 20 flame photometers). The remaining plasma volume was extracted 1 : 4 with chilled trichloracetic acid (12·5 % v/v) and the supernatant analysed for glucose, α-amino nitrogen and ammonia. Glucose was determined by the *o*-toluidine method of Hyvarinen & Nikkila (1962) using Sigma reagents

Sigma Chemical Co., St. Louis, MO.), α-amino nitrogen was determined by the ninhydrin method of Clark (1964) using glycine as a standard. Ammonia was determined by a micro-modification of the salicylate-hypochlorite method of Verdouw, van Echteld & Dekkers (1978).

The net branchial fluxes of Na^+, Cl^- and ammonia (in μequiv or μmol/kg/h) were calculated from changes in their respective concentrations in the water. Net branchial H^+ flux was determined as the difference between the apparent H^+ uptake (apparent base loss) and the ammonia excretion by procedures described in McDonald & Wood (1981). In this technique, apparent H^+ uptake is determined by the change in the titratable alkalinity of the water. Water titrations were performed within 12 h of collection on aerated, 10 ml water samples thermostatted to the experimental temperature.

Table 2. *Resting branchial, renal and blood acid-base and ion parameters (means ± one S.E.M., N = 13) in rainbow trout*

pH = 7·4 and T = 16 ± 1 °C. Values determined 36–48 h following surgery.

Blood		Gill		Kidney	
pHa	7·78 (± 0·04)	$J_{net}^{H^+}$ (μequiv/kg/h)	−200 (± 47)	Total acid excretion (μequiv/kg/h)	9 (± 2)
$[HCO_3^-]$ (mequiv/l)	5·6 (± 0·7)	J_{net}^{Amm} (μmol/kg/h)	−419 (± 47)	Ammonia excretion (μmol/kg/h)	5 (± 1)
$[Na^+]$ (mequiv/l)	145 (± 4)	$J_{net}^{Na^+}$ (μequiv/kg/h)	+ 67 (± 24)	Na^+ excretion (μequiv/kg/h)	31 (± 4)
$[Cl^-]$ (mequiv/l)	132 (± 4)	$J_{net}^{Cl^-}$ (μequiv/kg/h)	+ 52 (± 35)	Cl^- (μequiv/kg/h)	18 (± 4)

The endpoint of the titration was a pH of 4·0 and the titrant employed was 0·02 N-HCl. It should be pointed out that a net branchial H^+ flux would be the movement across the gills of any of the following: H^+, NH_4^+, HCO_3^- or OH^-. While it is not possible to distinguish between these forms all are equivalent in terms of the acid-base status of the animal.

Urine pH and titratable acidity (TA-HCO_3^-) were determined immediately after collection as described in Wood & Caldwell (1978) and McDonald & Wood (1981). TA-HCO_3^- was determined as a single value in the double titration procedure recommended by Hills (1973). Titrants were 0·02 N-HCl and 0·02 N-NaOH, and the final end point of the titration was the blood pH value prior to acid exposure. Total renal acid output was calculated as the sum of the titratable acid efflux (TA-HCO_3^- × urine flow rate) and the ammonia efflux (NH_4^+ × urine flow rate).

RESULTS

Measurements of blood acid-base and ion parameters and branchial and renal ion and acid fluxes for rainbow trout in circumneutral soft water (Ca^{2+} = 243 μequiv/l) are listed in Table 2. All blood measurements were in the relatively narrow range of values previously reported for trout acclimated to a similarly low calcium environment (McDonald *et al.* 1980) and on that basis have been judged as normal. Under these

conditions all animals exhibited a net excretion of H^+ and a net uptake of Na^+ and Cl^-. The H^+ excretion occurred at both the gills and kidney but the gills accounted for over 95 % of the total. A similar partitioning for ammonia excretion was evident (Table 2). The animals were in positive ion balance prior to acid exposure as the net branchial influx of sodium and chloride exceeded renal losses by approximately two fold. This ion and acid balance was quantitatively similar to that previously reported for this species in hard water (Ca^{2+} = 1930 μequiv/l; McDonald & Wood, 1981). While this may reflect some residual disturbance due to surgical procedures and repetitive blood sampling there was at least no apparent additional disturbance due to the reduction in environmental Ca^{2+} levels.

When these fish were subsequently exposed to pH 4·3 (Table 1) a substantial mortality ensued. By 96 h, 70 % (9 out of 13) of the fish had died. This contrasts with acid exposure in hard water, where mortality was only 29 % by this time (McDonald & Wood, 1981); a difference which is largely due to the 10-fold lower Ca^{2+} levels in the present study since the concentration of H^+ and other ions were similar in the two studies (Table 1). This much higher mortality necessitated analysing the data in a different fashion than previously; the approach adopted here has been to treat the survivors and the non-survivors essentially as two separate groups. Statistical comparisons are difficult with this approach, nevertheless some insights into the toxic effects of low pH are possible.

Acid exposure prompted immediate and substantial branchial losses of sodium and chloride in both surviving and non-surviving animals (Fig. 2). In survivors (open bars, Fig. 2) branchial ion losses progressively fell over the first 48 h of exposure to about 40 % of initial values and remained at these levels for the following 48 h. A similar decrease was observed in non-survivors (shaded bars, Fig. 2) but by 48 h there was virtually complete mortality (one animal died 12 h later). In non-survivors the net losses of Na^+ and Cl^- were more substantial than in survivors, particularly over the initial 10 h flux period (Fig. 2A, B). As a result there was a more rapid decline in plasma levels in this group (Fig. 3A, B). Eventually, however, the survivors sustained slightly larger total ion losses than the non-survivors (Fig. 4). The former lost about 25 % of their exchangeable pools of Na^+ and Cl^- (estimates based on measurements of radio-sodium space and radio-chloride space of 300 ml/kg; D. G. McDonald, unpublished results) while the latter lost only about 20 %. Because of variability these losses were not significantly different. The plasma ion losses (Fig. 3A, B) were, however, significantly greater in survivors (by paired '*t*' test).

Accompanying the ion losses was an initially large net uptake of H^+ at the gills (Fig. 2D) but again this declined with time in both groups. In survivors the net movement of H^+ was negligible from 48–96 h. Indeed, the total net H^+ uptake over the 96 h period was of relatively low magnitude as it caused virtually no disturbance in survivors to either plasma pH (Fig. 3D) or HCO_3^- (Fig. 3C). In non-survivors a blood acid-base disturbance had not developed by 24 h, but by 48 h the status of the group was uncertain. Only two fish could be sampled at this time via the indwelling catheters; one animal was severely acidotic (pH = 7·380) the other was not; the remainder could be sampled only by cardiac puncture; a procedure which is inadequate for acid-base analysis (cf. Holeton *et al.* 1980).

Acid exposure prompted a gradual increase in branchial ammonia excretion (Fig.

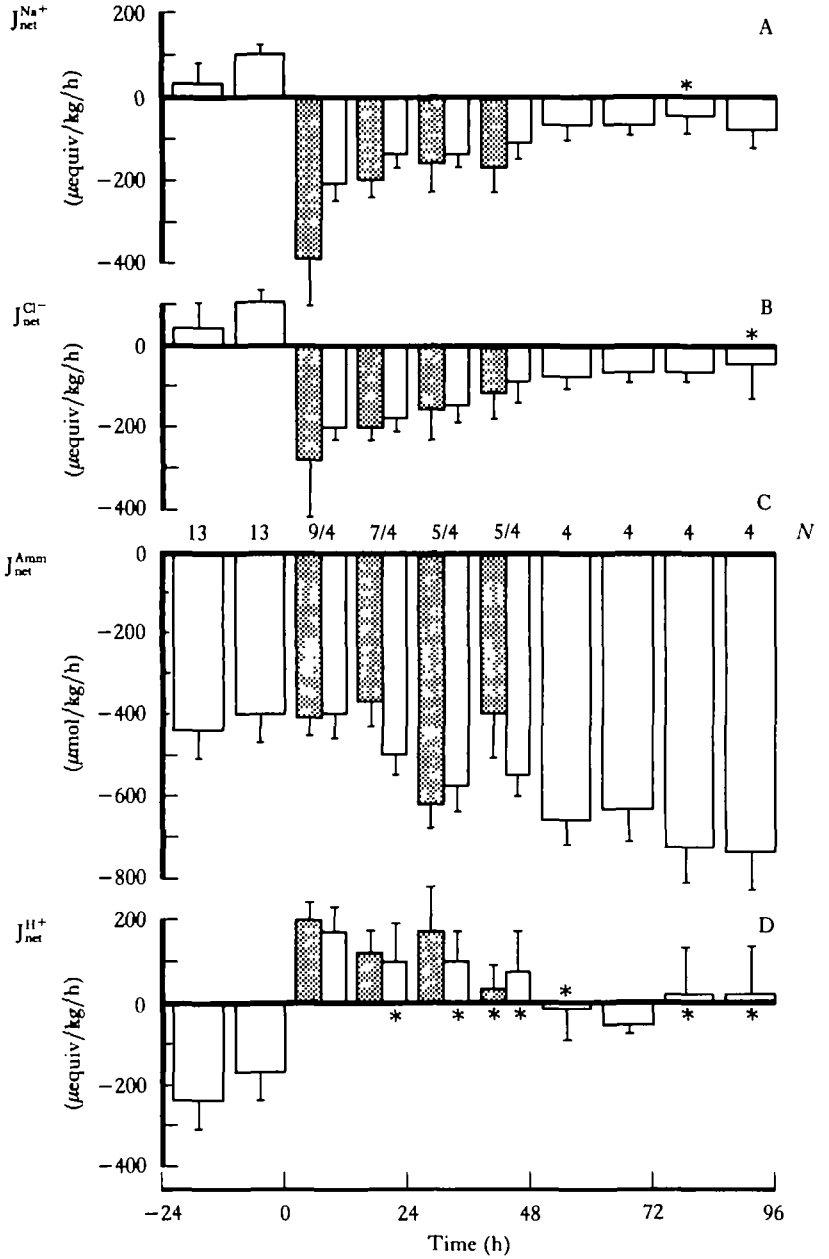

Fig. 2. Branchial net ion and acid fluxes (means±one S.E.M.) in rainbow trout at circumneutral pH (pH 7·4, −24 h to 0 h) and acid pH (pH 4·3, 0 to 96 h) at 16±1 °C. (A) $J_{net}^{Na^+}$, net sodium flux. (B) $J_{net}^{Cl^-}$, net chloride flux. (C) J_{net}^{Amm}, ammonia excretion. (D) $J_{net}^{H^+}$, net H^+ flux. Shaded bars are non-survivors ($N = 9$ declining to 0), open bars are survivors ($N = 4$). Asterisks indicate means not significantly different from zero ($P > 0{\cdot}05$ by 't' test).

2C) in survivors to levels about two-fold higher than pre-exposure levels. Similarly, in non-survivors there was an initial increase in ammonia excretion but this was followed by a significant decline (by paired 't' test) from 36–48 h. This appears to be

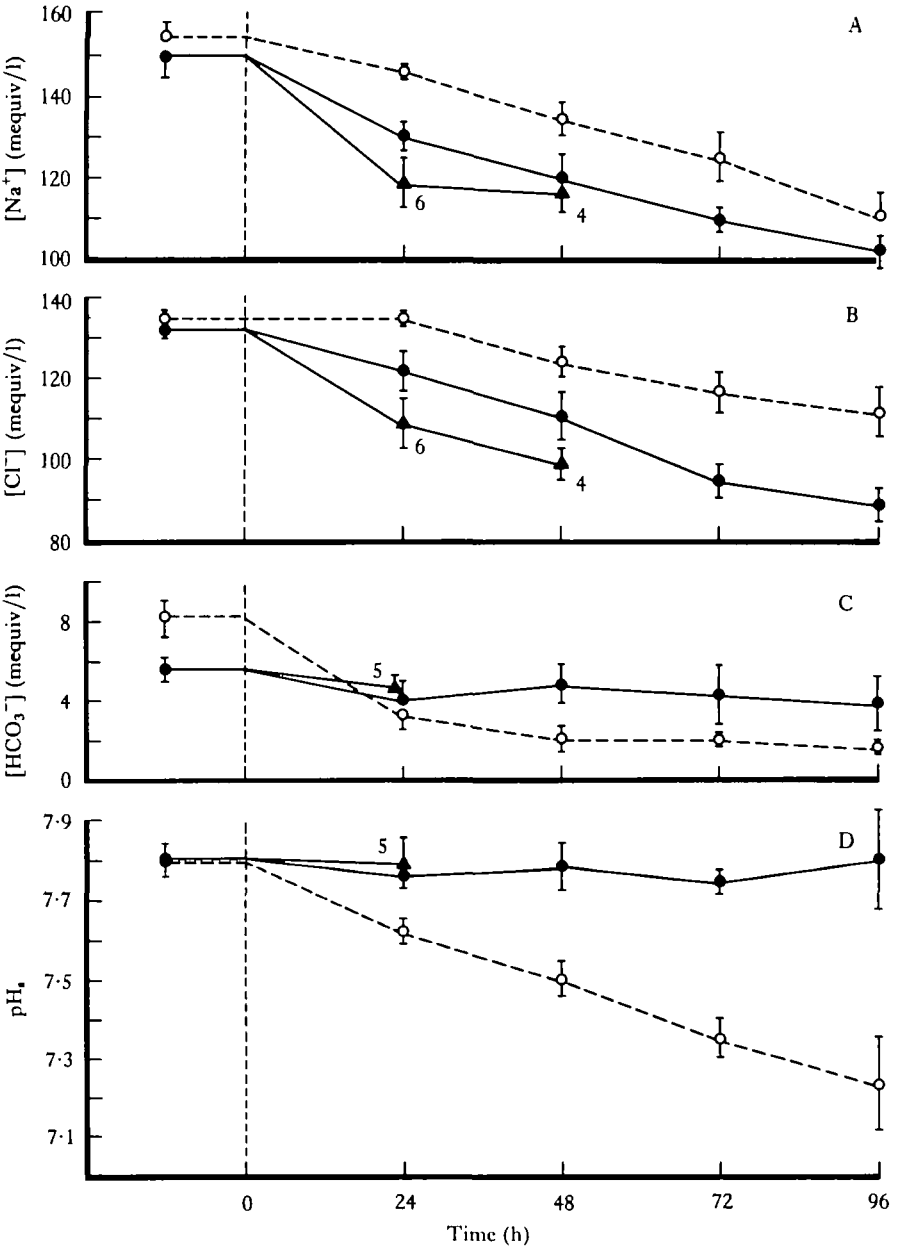

Fig. 3. Arterial plasma ion and acid-base state (means±one S.E.M.) in rainbow trout prior to and during low pH exposure (pH = 4·3). (A) sodium; (B) chloride; (C) bicarbonate; (D) pH. Solid lines indicate survivors (solid circles, $N = 4$) and non-survivors (triangles, N indicated on figure). Values for fish exposed to acid in hard water (pH 4·2 at Ca^{2+} = 1930 μequiv/l) are indicated by dotted lines ($N = 10$ at t = 0, declining to 7 at 96 h; data from McDonald & Wood, 1981).

one of the characteristics of incipient acid mortality; four out of five animals in this group died near or at the end of this period, the fifth died about 12 h later.

These branchial fluxes are compared to those found in high calcium water (McDonald & Wood, 1981) in Fig. 4, where total cation losses (i.e. $Na^+ + K^+$) are

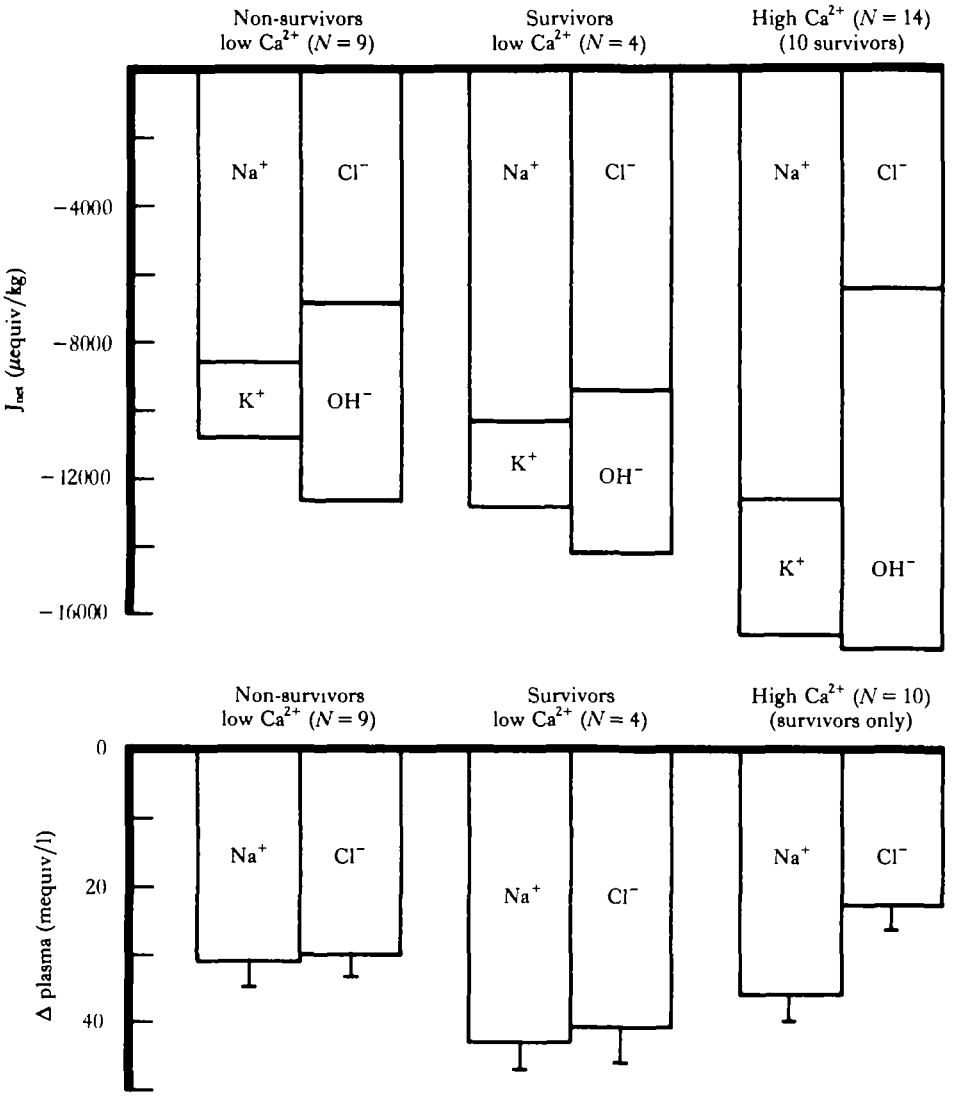

Fig. 4. (A) Total branchial ion loss (J_{net}) during acid exposure (pH = 4·3) in the rainbow trout. Losses arranged as total cation ($Na^+ + K^+$) *vs* total anion ($Cl^- + OH^-$). (B) Total plasma Na^+ and Cl^- losses (means±one S.E.M.). For survivors in both low Ca^{2+} (223 μequiv/l) and high Ca^{2+} (1930 μequiv/l) the duration of acid exposure was 96 h, for non-survivors in low Ca^{2+} the median duration of acid exposure was 42 h. Note that branchial ion losses in high Ca^{2+} were measured on two groups of fish (N = 6 + 8) and include contributions from four fish that died. The plasma ion losses for this group are for survivors only. High Ca^{2+} data are from McDonald & Wood (1981).

plotted *vs* total anion losses (i.e. $Cl^- + OH^-$) for each group of animals. The net H^+ influx has been regarded as a net OH^- efflux for this purpose. Since net cation movements must equal net anion movements for charge balance to be maintained, any discrepancy must be due to the flux of an unmeasured ion or to measurement error. Fortunately, these factors can be regarded as relatively minor since the discrepancies

were about 10 % or less of the total loss in all cases. This analysis reveals that ther were two major effects of water calcium. Firstly, chloride losses decreased relative to sodium losses with increasing calcium. Sodium and chloride losses were nearly equimolar in soft water, while in hard water, sodium losses were about twice the chloride losses. These patterns of branchial ion loss were reflected in the plasma ion losses (Fig. 4B). However, it should be noted that there is an apparent anomaly in this comparison. The plasma ion losses in hard water (Fig. 4B) were slightly lower than those in soft water but the branchial ion losses were higher. This stems from the fact that the branchial fluxes in hard water were determined as a single value on groups rather than individuals and include ion fluxes from four fish that died later in the experiment. Judging from the decline in plasma ion levels in these fish, they contributed a disproportionately large amount to the total ion losses of the group. The second major effect of calcium was that H^+ uptake was more than doubled in hard water. Again, this was reflected in the changes in plasma pH. The pH disturbance in soft water acid-exposed fish was negligible, while in hard water, plasma pH declined by about 0·5 units (Fig. 3D).

The renal response to acid exposure also showed the pronounced influence of water calcium (Fig. 5). Here the data from all fish have been pooled to calculate the means, since no marked differences between survivors and non-survivors were noted. At circumneutral pH the urine flow rate (Fig. 5D) in soft water was virtually identical to that found previously in hard water (dotted lines, Fig. 5). Similarly, there was a progressive decline in urine flow with acid exposure. However, the decline was more rapid in soft water and there was not the initial significant increase which had occurred in hard water. This pronounced reduction in flow limited renal Na^+ and Cl^- losses (Fig. 5A, B) to levels much lower than that seen previously. In hard water the renal losses by 96 h amounted to 23 % of the total body loss (McDonald & Wood, 1981), while in soft water the renal losses by this time were only 11 % of the total. Furthermore, there was no change in renal acid excretion (i.e. $TA\text{-}HCO_3^- + NH_4^+$; Fig. 5C) with acid exposure in soft water. This was in marked contrast to the renal response in hard water (Fig. 5C), where acid excretion increased to a level 15-fold higher than pre-exposure levels.

The second series of experiments (Table 3) was designed to examine the physiological effects of low pH at an even further reduction of environmental calcium levels ($Ca^{2+} = 69$ *vs* 223 μequiv/l, Table 1). In this series, mortality was more rapid and higher than in the first series. By 48 h, when the experiment was terminated, 10 out of 12 fish (83 %) had died. At 40 h, four fish were still alive and their measured blood parameters and branchial fluxes are listed in Table 3. Two of these fish were in visible distress at this time, showing loss of equilibrium, elevated ventilation rates and characteristic discolouration. All were subsequently resampled at 44 h. These values (Table 3) clearly show the progression of the acid toxicity syndrome.

The plasma ionic disturbance at 40 h was virtually identical to that evident in non-survivors (first series) at about this time (Fig. 3A, B). Branchial Na^+ and Cl^- losses were, however, much higher and were more typical of the initial efflux rates in non-survivors (Fig. 2A, B). At these elevated rates of ion loss there was a significant depression in plasma ion levels over the next 4 h (Table 3).

The fish had also developed a definite acid-base disturbance by 40 h, although this

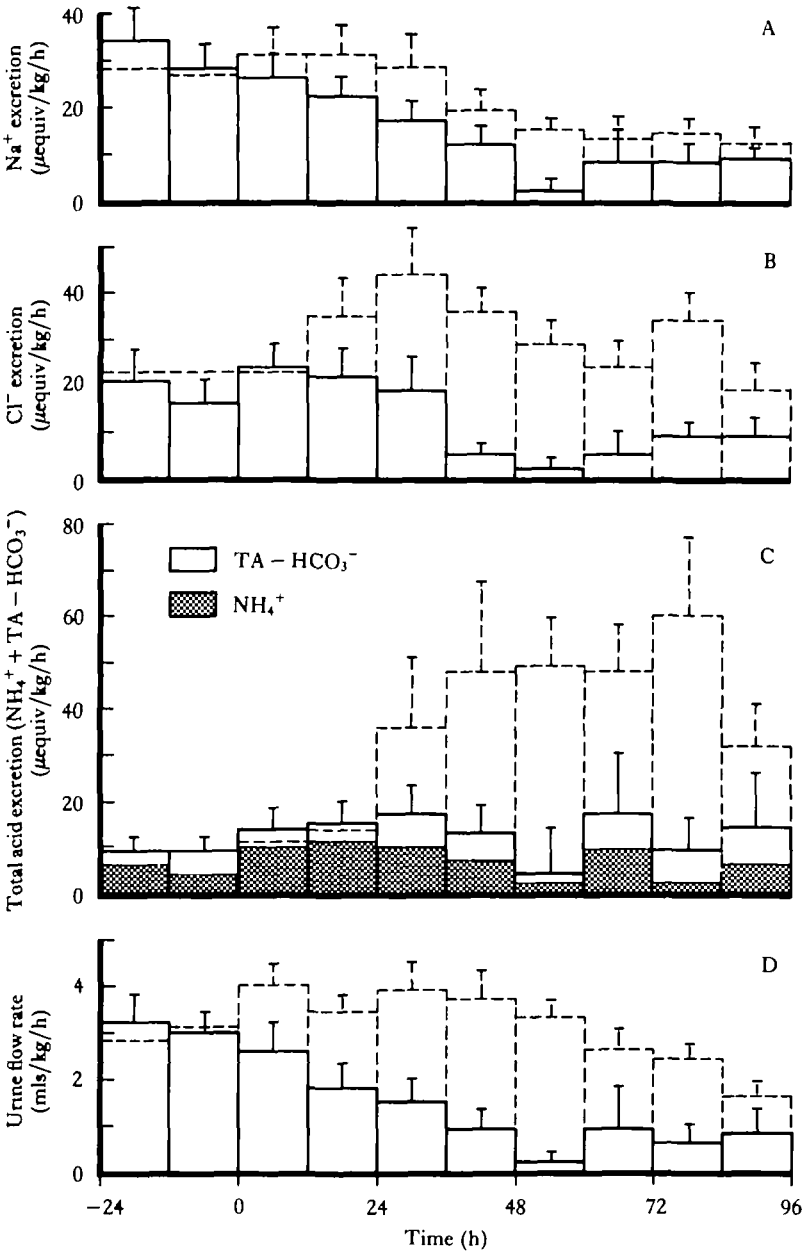

Fig. 5. Renal excretion in rainbow trout at circumneutral pH (pH = 7·4, −24 h to 0 h) and at acid pH (pH 4·3, 0 to 96 h). (A) sodium excretion rate. (B) chloride excretion rate. (C) total acid ($NH_4^+ + TA - HCO_3^-$) excretion rate. (D) urine flow rate. Values are means±one S.E.M. ($N = 13$ at 0 h declining to 4 at 96 h). Dotted lines are means (±one S.E.M., $N = 14$ at 0 h declining to 10 at 96 h) obtained for rainbow trout in high Ca^{2+} water at pH = 4·2; data of McDonald & Wood, 1981.

was not as large as that seen in hard water by this time (Fig. 3D). Four hours later this disturbance had progressed further (Table 3, +44 h) but this was partially attributable to a respiratory acidosis resulting from a significant elevation in P_{a,CO_2} over this period.

Table 3. *Branchial flux and blood measurements (means ± one S.E.M., N = 4) in extreme soft water (Ca^{2+} = 69 μequiv/l)*

All values at 40 h were significantly different from time 0 values ($P < 0.05$ by paired '*t*' test) except P_{a,CO_2}. Asterisks indicate means significantly different from 40 h values ($P < 0.05$, by paired '*t*' test).

P_{a,CO_2}	t = 0 h at pH_w = 7·4	+40 h exposure to pH_w = 4·2	+44 h exposure pH_w = 4·2
pHa	7·86 (± 0·04)	7·58 (± 0·10)	7·39 (± 0·14)*
P_{a,CO_2} (mm Hg)	2·5 (± 0·3)	2·4 (± 0·3)	5·0 (± 1·3)*
HCO_3^- (mequiv/l)	6·6 (± 0·2)	3·3 (± 1·1)	4·9 (± 0·8)*
Na^+ (mequiv/l)	148·5 (± 4·6)	114·8 (± 6·1)	110·0 (± 5·8)*
Cl^- (mequiv/l)	137·0 (± 1·5)	97·3 (± 8·4)	91·5 (± 8·0)*
osmolarity (mOSM)	300·3 (± 3·6)	276·3 (± 7·8)	266·4 (± 4·8)*
ammonia (mmol/l)	0·31 (± 0·02)	0·52 (± 0·05)	0·46 (± 0·01)
α–amino N (mg N/l)	70·5 (± 1·9)	159·1 (± 35·7)	162·8 (± 39·0)
glucose (mmol/l)	2·8 (± 0·2)	13·0 (± 1·9)	11·7 (± 3·3)
plasma protein (gm/l)	18·6 (± 0·8)	31·7 (± 2·6)	31·9 (± 2·6)
haematocrit (%)	17·8 (± 4·2)	31·3 (± 30)	35·1 (± 5·6)
		+40 h to +42 h	+42 h to +44 h
$J_{net}^{Na^+}$ (μequiv/kg/h)	+ 16 (± 28)	−360 (± 56)	−340 (± 93)
$J_{net}^{Cl^+}$ (μequiv/kg/h)	− 7 (± 34)	−319 (± 63)	−303 (± 99)
J_{net}^{Amm} (μmol/kg/h)	−341 (± 30)	−463 (± 11)	−349 (± 16)

Correlated with the depression in plasma ion levels was a reduction in plasma osmolarity. But, whereas Na^+ and Cl^- levels had fallen by 34 and 40 μequiv/l respectively at 40 h (Table 3), osmolarity had only fallen by 24 mOSM or about 50 mOSM less than predicted from ionic changes. This was due to sigificant increases in the concentrations of glucose (Δ = 11 mmol/l), amino acids (Δ = 90 mg N/l ≃ 7 mmol/l), plasma protein (Δ = 13 gm/l) and ammonia (Δ = 0·21 mmol/l) in that order of importance.

DISCUSSION

Interaction of environmental calcium with low environmental pH

It is evident from the present study and from extensive field observations (Leivestad & Muniz, 1976; Leivestad, Muniz & Rosseland, 1980; Muniz & Leivestad, 1980) that the principal effect on fish of chronically toxic acid exposure in soft water (pH 4·0–4·5 at $Ca^{2+} \leq 300$ μequiv/l) is an ionoregulatory disturbance. Furthermore, this disturbance arises almost exclusively from the net loss of ions at the gills (Figs 2, 4) and is exacerbated when calcium levels are reduced (Table 3). While disturbances to acid-base regulation and to tissue oxygen delivery do occur in acid environments (see Wood & McDonald, 1982 for review) these disturbances usually only predominate when elevated calcium (≥1000 μequiv/l) is combined with a lower external pH (pH ≤ 3·5, cf. Ultsch, Ott & Heisler, 1981); conditions which fish are unlikely to encounter in the wild. At realistic pH minima in soft water such disturbances are strictly secondary effects, occurring only after a severe electrolyte imbalance has developed and death is imminent (Table 3, this study; Table 4 of McDonald *et al.* 1980). Thus of central importance to understanding the effects of low pH on fish is

understanding how acid environments interfere with branchial ionoregulatory mechanisms and how these effects are modulated by external calcium.

The specific nature of this interaction is examined in more detail in our following study (McDonald, Walker & Wilkes, 1983) but it is at present possible to identify three major aspects. First, a decrease in the water calcium level at chronically toxic pH (pH 4·0–4·5) increases the rate of plasma ion loss (Fig. 2; McDonald *et al.* 1980; Leivestad, *et al.* 1980). This effect is most apparent at very low calcium concentrations where variations in calcium concentration become as important to ion balance as variations in pH. For example, an approximate doubling of the water calcium level (8·0–18·0 μequiv/l at pH 4·0) reduced plasma ion losses in brown trout, *Salmo trutta*, by as much as did halving the $[H^+]$ (pH 4·0–4·3 at 8 μequiv/l Ca^{2+}; Leivestad *et al.* 1980). Secondly, plasma chloride losses increase relative to plasma sodium losses with decreasing water calcium. In moderately hard water (Ca^{2+} = 1600 μequiv/l) the ratio of plasma Na^+ loss to Cl^- loss was 3·7:1 (McDonald *et al.* 1980); in soft water (Ca^{2+} = 223 μequiv/l) the losses were nearly equimolar (Fig. 4), and in extremely soft water (Ca^{2+} = 8 μequiv/l) the ratio was 0·8 Na^+: 1 Cl^- (Leivestad *et al.* 1980). Thirdly, decreasing the water calcium level substantially decreases the rate of acidification of the plasma at low pH such that below calcium levels of about 400 μequiv/l the blood pH disturbance was virtually negligible (Fig. 3; McDonald *et al.* 1980).

These effects on plasma ion and acid base balance are now corroborated to a large extent by the patterns of net ion and H^+ movement across the gills. The present study demonstrates that a reduction in water calcium increases net Cl^- loss relative to Na^+ and also decreases net H^+ uptake (Fig. 4). There is less clear evidence for an overall increase in branchial ion losses with decreasing Ca^{2+} but this, as pointed out in the Results, is obscured by methodological differences between the present study conducted in soft water and our previous study (McDonald & Wood, 1981) conducted in hard water. A recent, more thorough, investigation of ion fluxes at a pH of 4·0 in hard water (Ca^{2+} = 1000 μequiv/l; Booth, Jansz & Holeton, 1982) reported Na^+ and Cl^- net losses that were, on average, about 50% lower than those measured in soft water (Ca^{2+} = 243 μequiv/l) at a pH of 4·3 (Fig. 2). Furthermore, a reduction in calcium to 69 μequiv/l (Table 3) led to higher rates of ion loss at 40 h of acid exposure than seen at this point in 243 μequiv/l Ca^{2+} (Fig. 2).

Environmental calcium has well known effects on the ion and water permeability of fish gills (Potts & Fleming, 1971; Oduleye, 1975; Eddy, 1975; McWilliams & Potts, 1978; Pic & Maetz, 1981; Wendelaar Bonga & van der Meij, 1981). Thus it is tempting to speculate that the ionic disturbances which develop at low pH originate largely from calcium-modulated increases in the permeability of the gills to sodium and chloride; the latter increasing relatively more than the former with calcium reduction. This simple model would explain the patterns of ion loss described above. It would also explain the Ca^{2+}-dependent nature of the net branchial H^+ flux if two assumptions are made: that H^+, Na^+ and Cl^- are the principal diffusing ions at low pH, and that any active transport of sodium which may be occurring is of a similar intensity to that of chloride. Under these conditions the maintenance of charge balance would dictate that if Na^+ permeability was greater than Cl^- permeability then Na^+ efflux would be greater than Cl^- efflux and the difference would be made up by passive H^+ influx. If, on the other hand, Na^+ efflux equalled Cl^- efflux then H^+

influx must be much lower. Any influx of H^+ under this circumstance would have to be balanced by efflux of another cation (e.g. K^+ or Ca^{2+}) or by influx of another anion (e.g. SO_4^{2-}). This is undoubtedly a highly simplified view of the effect of low pH and calcium on the gills. It does not take into account possible Ca^{2+}-dependent disturbances to active ion transport (see McDonald *et al.* 1983) nor the likelihood of longer-term compensating adjustments in ionoregulatory mechanisms with continued acid exposure. Nevertheless, the hypothesis that the level of external calcium essentially controls the rate of passive H^+ entry across the gill is preferable to the alternative; an increasing rate of H^+ excretion with calcium reduction. This excretion would have to involve either an active ion exchange process, i.e., Na^+/H^+, Na^+/NH_4^+ exchange (Maetz, Payan & De Renzis, 1976) or the diffusional efflux of NH_4^+ (cf. Kormanik & Cameron, 1981). While both may be occurring to some extent at low pH it is very difficult to see how reducing the external calcium concentration would act as a further stimulus to either mechanism.

Adaptive responses to low pH

Low pH exposure prompted a number of responses from rainbow trout which can be considered as adaptive to the effects of acid stress. These responses were the progressive reduction in net salt losses (Fig. 2A, B), in net H^+ uptake (Fig. 2C) and in urine flow rate (Fig. 5D), and the pronounced increases in ammonia excretion at the gills (Fig. 2D) and in the blood concentrations of glucose and amino acids (Table 3).

Compensation of the ionic imbalance occurred to a large extent via the gills. Net Na^+ and Cl^- losses declined substantially, particularly in survivors (Fig. 2A, B) and most probably involved adjustment to both active ion transport and to ion permeability (McDonald *et al.* 1983). The decrease in urine production by the kidney played a small role in this compensation by further reducing renal ion losses by this normally minor route (Fig. 5A, B). However, the compensation was far from complete, even in survivors. Net ion loss continued throughout exposure and there was no evidence of recovery in plasma ion levels (Fig. 3). It is unlikely, in fact, that the survivors would have recovered if the acid exposure had been continued. Such recovery takes at least two weeks at even a mildly acid level (pH 6·0, *Salmo trutta*; McWilliams, 1980). Given the high mortality and substantial ion losses by 4 days of pH 4·3 exposure it is more probable that all fish would have eventually perished from the effects of uncompensated ion loss (see below).

Rainbow trout, on the other hand, seem to be able to make full compensation for H^+ uptake in soft water (Fig. 2D). Whether this was due to a progressive reduction in H^+ permeability (or adjustments in Na^+ and Cl^- permeability; see above) or was due to a progressive activation of H^+ excretion is uncertain. Superficially, the increase in ammonia excretion would seem to be important in this regard, as any NH_4^+ efflux would be equivalent to the excretion of a proton. NH_4^+ excretion (whether active or passive) cannot, of course, be ruled out and it is possible that the large pH gradient across the gills was responsible for activating ammoniogenesis as a means of defending acid-base balance. There are, however, two arguments against this possibility. First, McDonald *et al.* (1983) have shown that branchial ammonia excretion during acid exposure is not significantly affected by environmental calcium over a 90-fold range

n concentration (Ca^{2+} of 60–5700 μequiv/l) despite a pronounced effect of calcium on the net H^+ flux. This suggests that ammonia excretion does not play a major role in H^+ excretion during acid exposure. Second, it seems more likely that the increased ammoniogenesis was, in fact, a component of a general stress response involving the pituitary–interrenal axis.

Recently, Ashcom (1979) has shown that exposing brook trout to pH 4·0 caused an immediate and substantial increase in the plasma concentration of the interrenal hormone, cortisol. Cortisol is a well known index of stress in teleosts (Mazeaud, Mazeaud & Donaldson, 1977) and has manifold physiological effects including the stimulation of gluconeogenesis and protein catabolism (Butler, 1973). When cortisol is elevated, the plasma concentrations of glucose, amino acids and ammonia rise (Freeman & Idler, 1973; Chan & Woo, 1978) and there is an increase in branchial ammonia excretion (Chan & Woo, 1978). The presence of the identical phenomena in the acid-exposed rainbow trout (Table 3), thus strongly argues for a hormonal basis to the increase in ammonia excretion.

The question thus arises as to the role that these presumably cortisol-mediated responses may be playing in the adaptation of trout to acid stress. Current evidence suggests that the main benefit may be the mobilization of metabolic substrates required for fluid volume regulation (Assem & Hanke, 1981). In addition to their role as substrates however, plasma amino acids and, in particular, glucose served to reduce the depression in plasma osmolarity caused by branchial sodium and chloride losses (Table 3) and this, in the short term, may be the more important effect. Furthermore, cortisol may have played a direct role in the reduction of branchial salt losses since Ashcom (1979) has shown that chemical blockade of the cortisol response to acid exposure led to an increase in branchial sodium loss. A complicating factor, however, is the observation that acid exposure also stimulates excretion of the pituitary hormone, prolactin (Notter, Mudge, Neff & Anthony, 1976). Although this observation is based on histological evidence only, it may prove to be more significant as prolactin is regarded as the predominant hormone involved in minimizing ion losses in freshwater fish (Lahlou, 1980). Clearly, further work on the nature and extent of the hormonal involvement in adaptation to acid stress is required before this question can be resolved.

Ionic disturbances and mortality

From a study of a fish kill in the Tovdal river, Leivestad & Muniz (1976) reported that the lowest plasma Na^+ and Cl^- levels were found in fish dying from acid stress. This was also the case for the rainbow trout (Fig. 3A, B at 24 h) but the present data emphasize that it was the rapidity of the ion loss rather than the total amount lost which was important in determining lethality (Fig. 4). A similar observation was made by Packer & Dunson (1972) who showed that the rate of branchial Na^+ loss and mortality was accelerated by a reduction in pH (from 3·25 to 2·0) but that the total amount of Na^+ lost was reduced. These observations indicate that it is not the total ion depletion *per se* which is responsible for death but rather the secondary consequences of rapid branchial ion loss. Thus it is worthwhile to examine what these consequences are and how they lead to death.

Recently, Milligan & Wood (1982) proposed a sequence of physiological events

leading to death for which the present study now provides further evidence. This sequence is initiated by branchial ion loss whose immediate effect is to cause a decline in the osmotic pressure of the plasma (Table 3) and thereby cause an osmotic gradient across cell membranes. As a result the intracellular fluid space expands at the expense of the ECF (McDonald & Wood, 1981; Milligan & Wood, 1982). This, in turn, has three major effects: haemoconcentration, as indicated by a decrease in blood volume (McDonald *et al.* 1980; Milligan & Wood, 1982) and increases in haematocrit and plasma protein concentration (Table 4, also McDonald *et al.* 1980; Milligan & Wood, 1982); increased blood viscosity and pressure (Milligan & Wood, 1982); and a pronounced reduction in urine flow rate (Fig. 5). Superficially, the latter would seem unlikely, given both the increase in blood pressure and the relatively minor reduction in plasma osmotic pressure (5%, Table 3). However, it is the increase in plasma protein concentration (Table 3) which is of importance here. This increase will, in turn, lead to an exponential increase in plasma colloid osmotic pressure (Guyton, 1981) which will have a dual effect on the urine flow rate. Glomerular filtration rate will decline because of a decrease in net filtration pressure, and tubular reabsorption will increase because plasma protein would not be filtered. In any case, the important disturbances are the increase in blood viscosity together with the contraction of the blood volume. Cardiac work will increase, tissue oxygen perfusion will fall and severe tissue hypoxaemia, leading to death, will ensue.

Rapid ion loss rather than the total amount lost is the key to the above physiological sequence. With a more moderate rate of ion loss, hormonal responses to acid stress would have an opportunity to influence processes of fluid volume and salt regulation and would thereby increase the animals' tolerance of ion depletion. This would prolong survival during acid exposure but not for an indefinite period. Longterm survival would require the recovery of body salt levels, and the present study indicates that such recovery is unlikely to occur at low pH (i.e. $pH \leq 4{\cdot}5$) particularly in soft water. This, once again, emphasizes the critical role that environmental calcium will play in determining the status of fish populations in environments that are being progressively acidified.

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